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Seasonal Variations in Cardiac Sarcoplasmic Reticulum Activity in a Hibernator, the Richardson's Ground Squirrel (Spermophilus richardsonii)

by

Darrell David Belke

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE.

Department of Zoology

Edmonton, Alberta

Spring, 1989

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ISBN 0-315-52772-2

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NAME OF AUTHOR: DARRELL DAVID BELKE

TITLE OF THESIS: SEASONAL VARIATIONS IN CARDIAC SARCOPLASMIC RETICULUM ACTIVITY IN A HIBERNATOR, THE RICHARDSON'S GROUND SQUIRREL (Spermophilus richardsonii).

DEGREE: MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED: SPRING, 1989

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled SEASONAL VARIATIONS IN CARDIAC SARCOPLASMIC RETICULUM ACTIVITY IN A HIBERNATOR, THE RICHARDSON'S GROUND SQUIRREL (Spermophilus richardsonii) submitted by Darrell David Belke in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

(Supervisor) .....

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Date: December 16, 1988

ABSTRACT

Mammalian hibernation is a unique physiological state where animals can survive for days or weeks at a body temperature a few degrees above the freezing point of water and retain the ability to rewarm spontaneously in the cold without any exogenous heat source. Myocardial tissue must continue to function at low temperatures, circulating blood to deliver oxygen and nutrients to, and remove wastes from, vital (albeit metabolically depressed) organs. In the Richardson's ground squirrel it has been observed that during hibernation papillary muscle preparations demonstrate a greater peak force, and attain this peak force faster, than animals in the nonhibernating state. As tension development in striated muscle is dependent upon the concentration of activator calcium during excitation, and activator calcium concentration is controlled by the sarcoplasmic reticulum (SR), the observed mechanical characteristics of the hibernating myocardial tissue suggests that the SR exhibits certain seasonal alterations in its calcium regulating function. To define these changes, cardiac SR was isolated from hibernating ground squirrels and nonhibernating ground squirrels at different times of the year, for analysis of calcium ATPase activity, calcium uptake and accumulation, and SR functional efficiency in the form of the Ca/ATP coupling ratio. Hibernating SR preparations exhibited the highest calcium ATPase activity, the fastest rate of calcium uptake, the greatest level of calcium accumulation, and a Ca/ATP coupling ratio that was slightly higher than that of nonhibernating animals. The fall animals demonstrated rates of calcium uptake and calcium accumulation greater

than other nonhibernating groups indicating these animals were preparing for hibernation. Spring and summer animals had low rates of calcium uptake and accumulation, indicating that the changes which occur during hibernation are lost soon after emergence from hibernation in the spring and remain low until fall. These results also correlated well with the increased rate of muscle relaxation seen in hibernating papillary muscle preparations when compared to the nonhibernating state, and suggest that hibernating cardiac myocytes can utilize larger amounts of calcium for contraction. The high rate of SR calcium uptake also functions to restore low levels of intracellular calcium rapidly after contraction.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Lawrence C.H. Wang for his guidance in my studies on hibernation, and for his help in the preparation of this thesis. His breadth of knowledge on hibernation, and his ability to analyze problems and find solutions still continues to amaze me. I would like to thank my other committee members, Dr. William C. Mackay and Dr. Angelo Belcastro for their helpful suggestions towards my research, and to Dr. Michael Wolowyk for replacing Dr. Belcastro on my committee after his departure from the University of Alberta. I would like to thank the department of zoology for its financial support in the form of a teaching assistantship. Finally, I thank Michael Jourdan, Jeff Westly, Raymond Lee, Dawn MacArthur, Jonathan Lakey, Lorna Lee and all of the others, too numerous to mention, who have worked in Dr. Wang's Laboratory for the help and friendship they have given me over the years.

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I. GENERAL INTRODUCTION

Hibernation in mammals is a unique physiological state characterized by lethargy, a profound decrease in body temperature (Tb) and a greatly reduced heart and respiration rate (Lyman, 1982). Hibernation is not an abandonment of homeothermy, as the animals are capable of increasing their metabolic rate at lower temperatures to prevent freezing (Heller et al., 1978). This observation is also apparent from the fact that hibernators will rewarm themselves back to normal Tb at periods during the hibernation season (termed "arousal from hibernation"), using only endogenously produced heat. Maintaining a low Tb during harsh environmental conditions is a strategy for saving energy. Wang (1978) estimates energy savings as high as 88% for an animal using hibernation in comparison to its remaining euthermic at 37°C during the hibernation season. Clearly, as most nonhibernating mammalian species would not survive a prolonged state of depressed body temperature, hibernators must have evolved unique biochemical and physiological adaptations to survive at low body temperatures.

While skeletal and visceral smooth muscle can remain quiescent during deep hibernation (Tb = 2-5°C) the heart must remain active, circulating oxygenated and deoxygenated blood and nutrients to vital (albeit metabolically depressed) organs. In addition, the heart plays a critical role during arousal as blood serves as a conductor of heat, spreading it from metabolically active tissues, such as the brown adipose tissue, to other parts of the body. Several investigators have noted a superior cold tolerance in hearts isolated from hibernating

species in comparison to those from nonhibernating species (Covino and Hannon, 1959; Lyman and Blinks, 1959; Smith and Katzung, 1966: South and Jacobs, 1973; Burlington et al., 1976; Jacobs and South, 1976). Lyman and Blinks (1959) note that hearts isolated from hibernating species continued to beat at temperature approaching 0°C, while those from nonhibernating species ceased beating between 10-15°C. The difference in myocardial performance at low temperatures is partly due to the inability of nonhibernating species to maintain membrane potentials; Marshal and Willis (1962) found that the membrane resting potentials of atria from the hibernating and nonhibernating 13-lined ground squirrels decreased only slightly between 31-6°C, while that of the rabbit fell rapidly below 25°C, indicating the inability of the rabbit heart to maintain adequate ionic gradients across the cell membrane; gradients which are essential for cell survival.

While hibernating and nonhibernating species differ greatly in the mechanical and electrophysiological response of their cardiac tissue when challenged by low temperature, changes in myocardial performance, within a given hibernating species, may also occur between animals in the hibernating and nonhibernating phase. The maximum peak tension in papillary muscles isolated from hibernating hamsters and Richardson's ground squirrels is greater than that from animals in the nonhibernating phase (South and Jacobs, 1973; Zhou et al., unpublished observations). Papillary muscles from hibernating Richardson's ground squirrels also demonstrated an increased rate of tension development when compared to animals in the nonhibernating phase. In general the activity of the muscle, whether it be the amount of force generated, or the dynamics of

contraction and relaxation, is related to the amount of activator calcium (calcium which allows the formation of cross bridges in the "sliding filament" theory of muscle contraction) within the myocyte (Schouten et al., 1987). The differences in the mechanical response of papillary muscle between the hibernating and nonhibernating states indicate a greater intracellular calcium release in the former, based on both the increased rate and extent of contraction seen in that state. In striated muscle, calcium metabolism for contraction and relaxation is under the control of the sarcoplasmic reticulum (SR).

The SR is an internal network of membrane bound tubules, found in close proximity to the contractile elements in striated muscle, which release and sequester calcium for muscle contraction and relaxation, respectively. The amount and organization of SR differs between the three main muscle types, being well developed in skeletal muscle, less developed in cardiac muscle and poorly developed in smooth muscle (Allen and Seidel, 1986; Van Winkle, 1986). Morphologically the SR can be divided into longitudinal, or nonjunctional SR, and cisternal, or junctional SR (Van Winkle, 1986). Junctional SR is found in association with transverse tubules (T-tubules) and is thought to function primarily in calcium release, leading to muscle contraction during excitation contraction coupling (Jones et al., 1986; Meissner, 1986; Chu et al., 1987). The nonjunctional SR, found as a reticular network of membranes between the junctional SR and T-tubules, constitutes a major portion of the SR and is thought to function primarily in calcium sequestration, leading to muscle relaxation through a reduction in intracellular calcium concentration (Chu et al., 1987). Pharmacological evidence

also supports the morphological and functional divisions of the SR; for example, ryanodine inhibits junctional SR calcium release but has no effect on nonjunctional SR calcium uptake (Besch, 1985) (because of the pharmacological action of ryanodine, junctional and nonjunctional SR vesicles are termed ryanodine sensitive and ryanodine insensitive vesicles respectively).

Sequestration of calcium into the lumen of the SR is an energy consuming process performed by a 100,000 Da intrinsic pump protein called the calcium-stimulated magnesium-dependent ATPase (this protein will be hereafter referred to as the "calcium ATPase" in this thesis). The function of the SR organelle is characterized by its protein composition, with the calcium ATPase being the most abundant protein in the SR, representing as much as 60-70% of all SR proteins in skeletal muscle, and being found in all portions of the SR except at the cisternal SR/T-tubule interface (Campbell, 1986). The calcium ATPase pump is itself regulated by a 22,000 Da protein called phospholamban, which contains two different sites for phosphorylation by cAMP-dependent protein kinase and by calmodulin-dependent protein kinase (Lindemann et al., 1986). Phosphorylation of phospholamban increases the activity of the calcium ATPase, and subsequently the rate of calcium uptake. This type of modulation of SR calcium uptake is important for the inotropic effects of compounds such as catecholamines, which load the SR with extra calcium by increasing the calcium influxing across the sarcolemmal membrane and stimulating the SR to accumulate more calcium.

By the nature of its physiological role as a source of calcium for contraction, the SR is also a powerful regulator of intracellular

calcium homeostasis during relaxation in cardiac myocytes. Of the three main organelles for regulating intracellular calcium homeostasis in cardiac muscle, the sarcolemma (SL), mitochondria, and SR; the SR is the most important (Carafoli, 1985). Mitochondria, while possessing a large capacity for the accumulation and storage of calcium (due to the abundance of this organelle), have a relatively low affinity for calcium (K_m approximately 10 μM), indicating they cannot be readily employed to achieve the low levels of calcium seen in the resting heart cell (10^{-7} to 10^{-8} M). Mitochondria are likely to function only as a calcium buffer in disease states in which resting intracellular calcium concentrations are increased over prolonged time periods (Dhalla et al., 1982). The SL, like SR, has a high affinity for calcium (SL calcium ATPase K_m = 0.5 μM, Na^+/Ca^{2+} exchanger K_m = 2-20 μM; SR calcium ATPase K_m = 0.1-0.5 μM) and can be used to obtain low sarcoplasmic calcium concentrations. It is limited however, in its capacity to transport calcium as it represents only about 0.8% of the total calcium transporting membrane found within heart cells compared to the 12.1% estimated for SR (mitochondria account for most of the remainder) (Carafoli and Penniston, 1985). As the mechanical action of cardiac muscle is dependent upon transient changes in intracellular calcium, and as the SR is the most important organelle for calcium metabolism during contraction and relaxation, an understanding of SR function is therefore important in understanding muscle activity in animals in both the hibernating and nonhibernating states.

Regulation of intracellular calcium at low temperatures is important for cell survival, as calcium serves as a regulator of many

cell functions either directly, or through calcium-calmodulin interactions (Tomlinson et al., 1985). For example, red blood cells from nonhibernators such as the guinea pig, do not survive more than a few hours at 5°C because of excessive loss of intracellular potassium when subjected to low temperatures; in comparison, those from hibernators such as the 13-lined ground squirrel can survive more than 7 days at this temperature without a significant loss of potassium (see Wang and Wolowyk, 1988 for review). In the guinea pig RBC potassium is lost through a calcium sensitive potassium channel (Gardos channel) due to the inability of these cells to cope with the continually influxing extracellular calcium by removing it from the cells at low temperatures. Hibernator RBCs, on the other hand, can maintain their intracellular calcium concentrations by actively pumping out extracellular calcium entering the cell despite the low temperature thus preventing the activation of the Gardos channel.

Clearly, if strict intracellular calcium regulation is important for cell survival at low temperatures, then this presents a special challenge for tissues, such as cardiac muscle, which require calcium pulses for normal functioning. Kondo and Shibata (1984) suggest that the hibernating Asian Chipmunk has solved this problem by relying more upon the recycling of internal calcium, stored in the SR, than extracellularly derived calcium for myocardial contraction. The hibernating chipmunk myocyte has a reduced plateau phase amplitude during membrane depolarization in comparison to that seen in the nonhibernating state, resulting in a reduced slow inward calcium current and consequently, less extracellular calcium entering the cell (Kondo,

1986). This observation is supported by the decreased tension development seen in papillary muscles from hibernating Richardson's ground squirrels, as opposed to nonhibernating preparations, when exposed to 10^{-4} - 10^{-2} M extracellular calcium to potentiate contraction (Charnock et al., 1980a). This indicates that although the hibernating muscle is bathed in a high calcium concentration it does not enter the myocyte as would be expected and therefore will not increase tension development. Similarly, both the Asian chipmunk and the Richardson's ground squirrel demonstrate an abolished, or severely depressed, positive inotropic response to isoprenaline and noradrenaline when they are in the hibernating state (Charnock et al., 1980b ; Kondo, 1986). Furthermore, in the hibernating 13-lined ground squirrel (Spermophilus tridecemlineatus) the sensitivity of ventricular force generation to adrenergic agonists is decreased (Senturia et al., 1986). As mentioned previously, these compounds alter cellular metabolism of calcium. The decreased influx of calcium during sarcolemmal membrane depolarization and the decreased sensitivity to catecholamines and agonists in the hibernating state seen in the different species presented above, indicates a common adaptation is apparently occurring in these animals; an adaptation based on fundamental changes in intracellular calcium metabolism. As SR is the major organelle involved in cardiac myocyte calcium metabolism an analysis of its activity seasonally, during the hibernating and nonhibernating phases, will aid in understanding the physiological changes observed in cardiac tissue in hibernating species.

Cardiac muscle from hibernating animals demonstrates, in general, an increased rate and extent of force generation when electrically

stimulated even though calcium influx across the sarcolemmal membrane is reduced during excitation contraction coupling. This suggests that cardiac myocytes rely more on the internal recycling of activator calcium from the SR. The present study represents the first attempt ever to document the cardiac SR function in a hibernating species, the Richardson's ground squirrels (Spermophilus richardsonii). Included are measurements of seasonal differences in SR calcium ATPase activity, SR calcium uptake and calcium accumulation, and the Ca/ATP coupling ratio within the physiological temperature range of the hibernator (37-4°C). Together, these measurements will provide a functional understanding on how cardiac calcium homeostasis may be accomplished in a hibernator both in euthermic and under profoundly depressed Tb.

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II. ISOLATION OF SARCOPLASMIC RETICULUM AND THE MEASUREMENT OF CALCIUM ATPase ACTIVITIES.

A. Introduction

The study of cardiac SR function is hampered by the fact that heart tissue is not as rich in SR as skeletal muscle tissue, and by the presence of large amounts of mitochondria, which can also function in calcium accumulation. A highly purified vesicular fraction of SR is easily obtained from rabbit white (fast twitch) skeletal muscle due to the presence of relatively few mitochondria, or from canine cardiac muscle in which many grams of tissue can be processed to obtain a pure fraction of SR. In small animals, such as rats and ground squirrels, such procedures do not provide a contamination-free preparation because the heart tissue is limited to 1 or 2 grams. In spite of this problem, however, the study of cardiac SR activity in small animals is still possible because 1) calcium uptake by mitochondria can be inhibited by sodium azide (Solaro and Briggs, 1974; Panagia et al., 1985); and 2) calcium uptake by SR vesicles is greatly enhanced by the presence of a calcium precipitating anion such as oxalate or phosphate, while calcium accumulation by SL is not, thus allowing the differentiation between SR and SL (Levitsky, 1984).

Although SR activity has been studied in whole heart homogenates (Feher et al., 1980; Newman, 1980; Hess et al., 1984), partial purification of the SR fraction through differential centrifugation is more advantageous in that: a) the contractile elements, a source of additional ATPase activity, are removed from the sample by treatment with 0.6 M KCl (Campbell, 1986), b) harmful proteases and lipases, which

can destroy SR function, are removed by the centrifugation process, and c) the more concentrated SR preparation amplifies the SR activity in a given experiment by increasing the signal/unit protein ratio.

The only previous study on SR calcium ATPase activity in a hibernating species was performed on skeletal muscle from the 13-lined ground squirrel (Becker and Willis, 1974). In that study the authors concluded that the temperature sensitivity (Arrhenius plots) of the calcium ATPase activity of the 13-lined ground squirrel and the rat was similar. Although the authors did not report the physiological state of the ground squirrels (ie. hibernating vs. nonhibernating) they did note that during hibernation skeletal muscle is relatively quiescent and inactive, and postulated that a muscle which must remain active during hibernation might provide different results. During hibernation cardiac muscle remains active, demonstrating changes in mechanical properties when compared to the nonhibernating state (see the General Introduction).

The objectives of this study are to: a) assess the purity of the SR preparations at different physiological states (hibernating, winter spring, summer and fall) with respect to the other two major calcium sequestering organelles commonly co-isolated in the SR microsomal fraction - mitochondria and sarcolemmal membranes; and b) to examine basal and calcium ATPase activities among the preparations with respect to season and thermal sensitivities. To do this, cardiac SR preparations were made from Richardson's ground squirrels captured at different times of the year, and during hibernation in the laboratory.

B. Materials and Methods

Animals

Richardson's ground squirrels (Spermophilus richardsonii) were live trapped using National traps, baited with sunflower seeds, near Edmonton Alberta, Canada. The trapping season lasted from late March to early October. Animals used for seasonal studies were typically terminated within one week of capture (usually 48-72 hr), while others were housed at room temperature in shoe box cages in the Biological Sciences Building Laboratory Animal Services Center, or at the Ellerslie Animal Services Center under natural light conditions or 12L:12D, respectively. The animals were given water ad lib., and fed a mixture of rat chow and Purina dog food ad lib. which was supplemented once a week with sunflower seeds. The animals were weighed weekly to determine their state in the annual hibernation cycle. Those animals showing a rapid weight gain and plateau (indicative of animals approaching their hibernating phase) were transferred to a cold environment chamber at 7°C (4L:20D) to induce hibernation. Once the animals began to hibernate they were transferred to another cold environment chamber (5°C) kept in total darkness to maintain hibernation. As continuous observation would disturb animals, bouts of hibernation were followed using the sawdust method (Lyman, 1948). No hibernating animal was terminated unless it had successfully completed at least one hibernation bout, demonstrating it was not hypothermic by rewarming in the cold without the aid of an exogenous heat source.

The freshly trapped animals used in the seasonal studies were

classified according to the capture date as illustrated in Table 2.1. While the capture of spring and fall animals was greatly dependent on environmental conditions, attempts were made to obtain animals as close as possible to the established field dates for the emergence from (late March), and immergence into (late September-early October) hibernation, respectively. From mid April to mid June trapping was suspended to allow the weaning of juvenile animals to be completed.

Isolation of the SR Fraction

All animals were terminated by decapitation. Hibernating animals were terminated in, or near, the cold environment chambers to minimize any disturbance due to transportation and handling which might lead to arousal from hibernation. Hearts were quickly removed from the animals and washed in a beaker of cold isolation medium (5 mM NaN_3 and 10 mM $NaHCO_3$, pH 7.0 at 25°C). The hearts were trimmed of fat and atria, weighed, and the ventricles were homogenized in 25 ml of cold isolation medium in a VirTis 23 homogenizer at full speed for a duration of 7 seconds, 4 times, with 15 second rest intervals (Velema and Zaagsma, 1981). All glassware was prechilled on ice before use.

The microsomal fraction containing SR was isolated according to the differential centrifugation method of Entman et al. (1973) (Figure 2.1), which is modified from the method of Harigaya and Schwartz (1969), using a Beckman L8-70M ultracentrifuge and a 70Ti rotor which had been prechilled to 4°C before use. The salt washing solution contained 0.6 M KCl and 20 mM tris maleate (pH 6.8, 25°C), and the storage solution

Table 2.1 Seasonal trapping schedule for active nonhibernating animals, other than winter animals, used in the present study. During spring and fall, trapping sites were monitored weekly to obtain animals as closely as possible to the general times of emergence and immergence respectively.

SEASONAL TRAPPING SCHEDULE

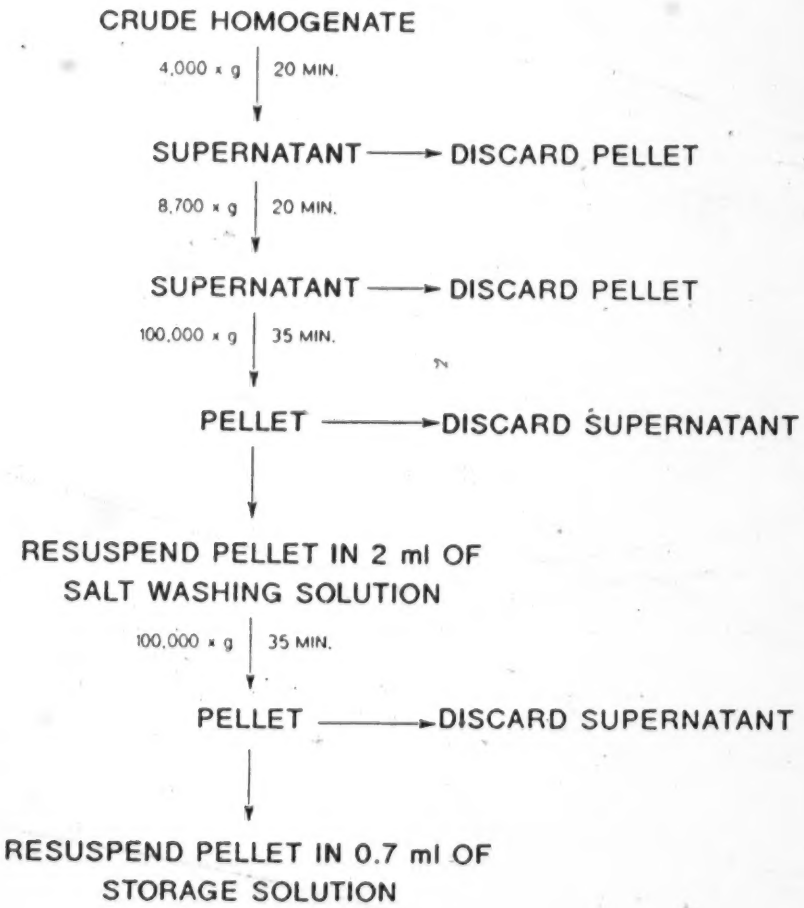
DATE CAUGHT:	SEASON:
mid March - mid April*	Spring Animals
mid June - mid August	Summer Animals
mid September - mid October**	Fall Animals

* Trapping is suspended between mid April and mid June until weaning of young is completed.

** The activity of the animals in the field was monitored weekly. Animals were trapped until squirrels disappeared for the winter.

Figure 2.1 The centrifugation scheme for the isolation of the sarcoplasmic reticulum (SR) enriched microsomal fraction used in this experiment. See text for solution composition.

DIFFERENTIAL CENTRIFUGATION SCHEME FOR CARDIAC SR ISOLATION FROM THE RICHARDSON'S GROUND SQUIRREL



contained 20 mM tris maleate (pH 7.1, 25°C). Resuspension of the pellet was achieved by the use of a glass tissue homogenizer (2 ml, Corning Glass Works) which was mounted on a motor. The final SR suspension was either placed on ice for immediate use, or frozen on dry ice and stored at -80°C in a Queue Cryostar freezer (Queue Systems, Parkersburg, W. Virginia). Freezing the SR preparation once, had no effect upon calcium uptake rates; thawed preparations were never refrozen and used again.

Assessment of Preparations

The concentration of protein in the microsomal (SR) preparation was determined by the Bradford method (Bradford, 1976) using the kits supplied by BIO RAD, or by preparing the protein reagent using Cromassie Brilliant Blue G (SIGMA); no differences were observed between using the commercial or laboratory prepared reagents. Bovine serum albumen (BSA) was used as the standard.

The extent of sarcolemmal (SL) contamination within the SR microsomal preparation was assayed using ouabain sensitive Na^+/K^+-ATPase as a SL marker enzyme. To determine the total extent (patent and latent) of ouabain sensitive Na+/K+-ATPase activity, the SR preparation was pretreated for 20 minutes at room temperature with 0.3 mg/ml sodium dodecyl sulphate (SDS) to allow the ouabain access the interior of the vesicles (Besch et al., 1976). The assay medium contained 100 mM NaCl, 10 mM KCl, 1 mM EGTA, 20 mM tris buffer (pH 7.40, 37°C), 4 mM Mg^{2+}-ATP, and 50-100 μg SR protein ± 1 mM ouabain. The tubes were pre-incubated at 37°C for 5 min in a shaking waterbath. The reaction was initiated by the addition of the SR protein, and allowed to continue for 10 min

before the addition of the colorimetric quenching reagent (Reagent III, see the next section for details). Inorganic phosphate liberated by the hydrolysis of ATP was measured by the method of Le Cocq and Inesi (1966) (see the next section for details).

The extent of mitochondrial contamination was measured using succinate cytochrome C reductase (Rabinowitz and de Bernard 1957), and cytochrome oxidase (Wharton and Tzagoloff, 1967). Succinate cytochrome C reductase activity was measured spectrophotometrically (550 nm) at 32°C in a 1 ml cuvette containing 30 μM cytochrome C (SIGMA TYPE III), 0.5 mM KCN, 40 mM potassium phosphate (pH 7.4), and 11 mM sodium succinate. The reaction was initiated by the addition of a known amount of SR protein. The rate of cytochrome C reduction was recorded using a Linear chart recorder. The cytochrome oxidase activity was measured in a cuvette containing 1 mM KH_2PO_4, to which 70 μl of 1% reduced cytochrome C (SIGMA TYPE III) was added. The reaction was initiated by the addition of a known amount of SR protein and was measured spectrophotometrically at 550 nm against a blank containing the above (excluding the SR) + 10 μl of 0.1 M potassium ferricyanide.

Measurement of Calcium ATPase activity by the Analysis of Inorganic Phosphate

Calcium ATPase activity of the SR was measured spectrophotometrically by determining the amount of inorganic phosphate cleaved from ATP present in the enzyme reaction mixture after 10 minutes of incubation. The molybdo-vanadate method of Le Cocq and Inesi (1966) was used to colorimetrically determine the amount of inorganic phosphate

in solution. The colorimetric reagent R-III contained 30 % (W/V) trichloroacetic acid, 0.017 M ammonium molybdate and 0.004 M ammonium vanadate. The reaction mixture contained 100 mM KCl, 100 mM sucrose, 0.5 mM $MgCl_2$, 5 mM oxalate, 5 mM NaN_3, 8 mM Mg^{2+}-ATP, 50 μM $CaCL_2$, and 20 mM tris maleate (pH 7.38). Total ATPase activity was measured in the presence of calcium, while basal (magnesium) ATPase activity was measured in the presence of 1 mM EGTA. Calcium ATPase activity was taken as the difference between total and basal activity. All test tubes contained 30 μg/ml A23187 (calcium ionophore) to maximize the calcium ATPase activity (this compound was also added to the assay medium for the basal ATPase activity to keep the two assay media as closely matched as possible). After a 3 minute preincubation of test tubes (1 ml of reaction mixture) in a shaking water bath (37°C), the reaction was started by the addition of a known amount of SR protein to the reaction mixture. The reaction was stopped after 10 min by the addition of a 1 ml aliquot of cold reagent R-III, and the test tubes were placed on ice. After spinning the samples at 2000 x g for 10 min, to remove any protein precipitate, a 1 ml aliquot was removed and diluted to 10 ml with double deionized water and measured at 350 nm in a PYE-UNICAM SP1800 visible/uv spectrophotometer against a double deionized water blank prepared in the same manner. Phosphate concentration was determined by comparing the absorbance obtained from the samples against a standard curve of 0.1 to 2.5 μmol/ml KH_2PO_4.

Measurement of Calcium ATPase Activities by the NADH Linked Enzyme Method

SR calcium ATPase activity was determined spectrophotometrically by continuously monitoring the oxidation of NADH at 340 nm. ATP hydrolysis was linked to the oxidation of NADH through the reaction sequence shown in Figure 1.2. Temperature control of the cuvette was achieved by a circulating water bath hooked into the cuvette holder in the spectrophotometer. The reaction mixture contained 100 mM KCl, 5 mM NaN_3, 100 mM sucrose, 100 mM KH_2PO_4, 2.5 mM Phospho(enol)pyruvate (SIGMA), 0.2 mM NADH, 9 units/ml pyruvate kinase (SIGMA), 12 unit/ml lactic dehydrogenase (SIGMA), 8 mM Mg^{2+}-ATP and 15-30 µg of SR protein. The reaction was started by the addition of the SR protein. As in the previous experiment calcium ATPase activity was taken as the difference between total (in the presence of calcium) and basal (in the presence of EGTA) activities. Because ATPase activity was monitored continuously data were analyzed over a time course and as initial rates.

All data were analyzed using an unpaired t-test with $p < 0.05$ being significant.

C. Results

Assessment of Preparation

Results from the mitochondrial marker enzyme assay are shown in Table 2.2. The succinate cytochrome C reductase assay indicates a mitochondrial contamination of 4-8% for all groups (based on an activity of 0.96 µmol cytochrome c/min/mg protein for beef heart mitochondria

Figure 2.2 The reaction scheme for the NADH-linked enzyme assay. Disappearance of NADH was measured spectrophotometrically at 340 nm. The stoichiometric ratio between ATP hydrolysis and the oxidation of NADH is 1:1. The phospho(enol)pyruvate (PEP)/pyruvate kinase system functions in ATP regeneration.

NADH LINKED ENZYME ASSAY FOR THE MEASUREMENT OF SR Ca-ATPase ACTIVITY

$$\underset{\text{(OUTSIDE)}}{Ca} + Mg\text{-}ATP \xrightarrow{SR\ Ca\text{-}ATPase} ADP + Pi + \underset{\text{(INSIDE)}}{Ca}$$

$$ADP + PEP \xrightarrow[KINASE]{PYRUVATE} ATP + PYRUVATE$$

$$PYRUVATE + NADH \xrightarrow[DEHYDROGENASE]{LACTATE} LACTATE + NAD$$

Table 2.2 Succinate cytochrome c reductase and cytochrome oxidase mitochondrial marker enzyme activities are shown here along with the estimated percent contamination for each group. Although the results are variable, both markers indicate low mitochondrial contamination in the different preparations. Apart from the summer group being significantly higher than the fall and winter groups in the succinate cytochrome C reductase assay, no statistical differences were found between the different groups for either marker enzymes. Shown is mean ± standard error.

MITOCHONDRIAL ENZYME ASSAY

	n	SUCCINATE CYTOCHROME C REDUCTASE	CYTOCHROME OXIDASE
HIBERNATING	5	0.074 ± 0.015 (7.7%)	0.117 ± 0.034 (9.4%)
SPRING	4	0.050 ± 0.006 (5.2%)	0.134 ± 0.025 (10.7%)
SUMMER	4	0.061 ± 0.001* (6.4%)	0.159 ± 0.043 (12.7%)
FALL	6	0.047 ± 0.004 (4.9%)	0.122 ± 0.007 (9.8%)
WINTER	4	0.041 ± 0.001 (4.3%)	0.096 ± 0.012 (7.7%)

(μmol cytochrome C/min/mg protein)

(Chamberlain et al., 1983)), while the cytochrome oxidase enzyme marker indicates a contamination of 8-13% for all groups (based on an activity of 1.247 μmol cytochrome C/min/mg protein for rat heart mitochondria). Although not statistically significant, the hibernating group had the highest level of enzyme activity in the first assay, while the summer group had the highest level in the second assay. In the succinate cytochrome C reductase assay the activity of the summer group was significantly higher than the fall and winter groups ($p < 0.05$), however the general level of mitochondrial contamination in all groups was low. The activity of the SL marker enzyme, ouabain sensitive Na^+-K^+ ATPase, is shown in Table 2.3. While the level of this marker enzyme was highest in the hibernating group it was not significantly different from the other nonhibernating groups. The marker enzyme activity was low in comparison to the 180 μmol Pi/mg/hr reported for this enzyme marker in purified SL in rats (Velema and Zaagsma, 1981) and canine SL activity of 88.5 μmol Pi/mg/ hr (Jones et al., 1980), indicating the level of SL contamination in the SR preparations ranged from roughly 5-10% maximum. The activity of this marker enzyme reported here is within the range reported elsewhere in the literature for isolation procedures based on the Harigaya and Schwartz (1969) method of SR microsomal extraction (Besch et al., 1976; Ganguly et al., 1983). The yield of SR protein per gram of heart tissue is shown in Figure 2.3., with the summer group giving the highest protein yield (1401 μg/g) and the winter group giving the lowest protein yield (895 μg/g) per gram ventricular weight. Only the winter group was statistically different ($p < 0.05$) from the other

Table 2.3 Ouabain sensitive Na^+-K^+ ATPase sarcolemmal marker enzyme. No statistically significant differences were observed between the different groups. All sarcoplasmic reticulum preparations used in this assay were pretreated with sodium dodecyl sulphate to unmask any latent ouabain sensitive Na^+-K^+ ATPase activity. Shown is mean $\pm$ standard error.

SARCOLEMMAL ASSAY

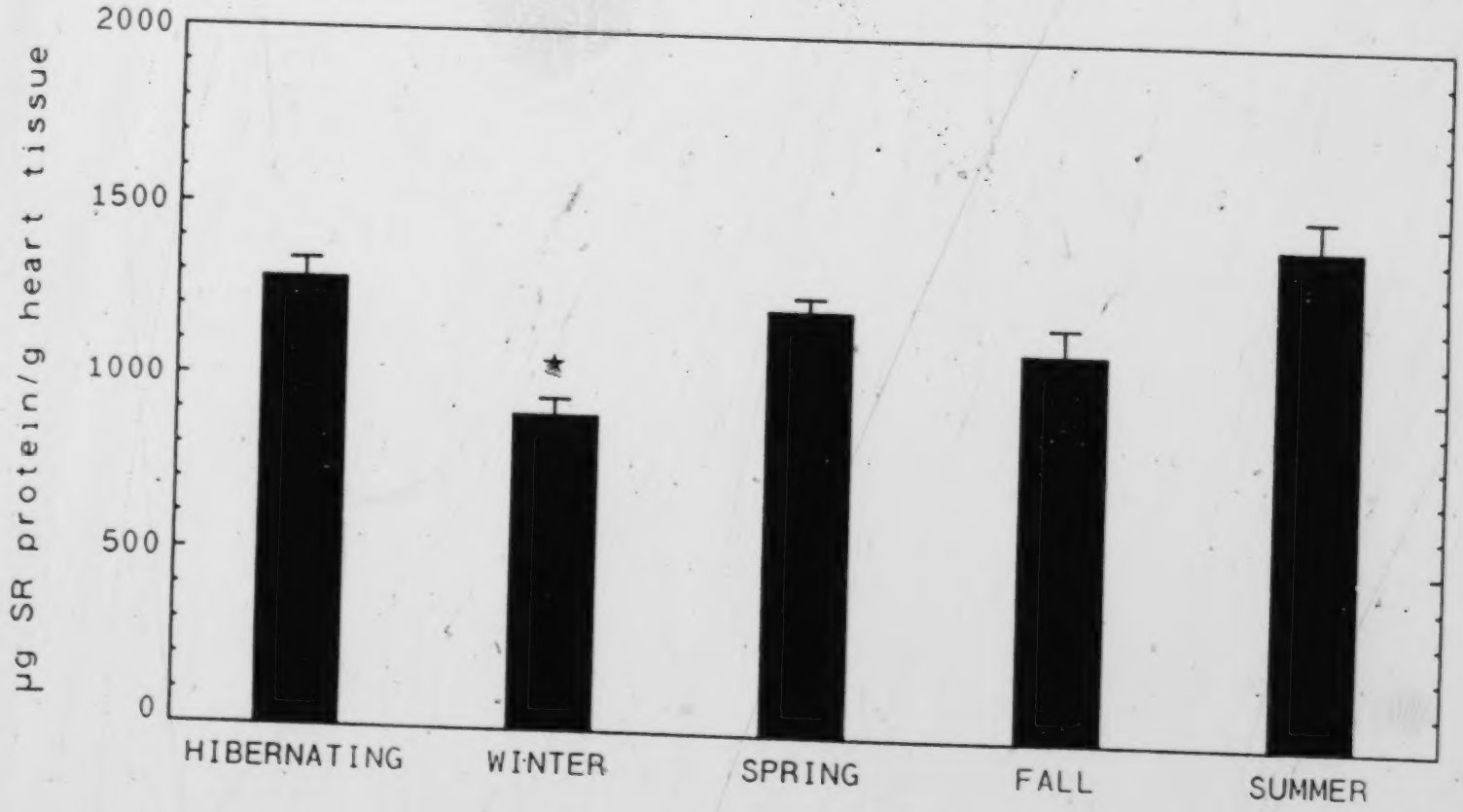
	n	OUABAIN SENSITIVE $Na^+ - K^+$ ATPase ACTIVITY
HIBERNATING	5	9.39 ± 2.48
SPRING	5	6.25 ± 1.07
SUMMER	4	7.40 ± 2.48
FALL	4	8.18 ± 3.49
WINTER	4	6.60 ± 2.26

(Decrease in μmol ATP hydrolyzed/mg protein/hr)

(Results reflect patent and latent activity)

Figure 2.3 Seasonal variation in sarcoplasmic reticulum protein yield. n = 27 for hibernating group, n = 21 for winter group, n = 34 for spring group, n = 15 for fall group and n = 17 for summer group. The winter group was significantly lower ($p < 0.05$) than all other groups (star). Bars represent mean ± standard error.

SEASONAL VARIATION IN SR YIELD



groups. The SR protein yield of 0.9-1.4 mg/g heart for the ground squirrel groups is approximately what is obtained by Entman et al. (1973) using dog heart.

Calcium ATPase Activities

Calcium ATPase and magnesium (basal) ATPase activities for the different preparations, as measured by the liberation of Pi from the hydrolysis of ATP at 37°C, are shown in Table 2.4. All measurements were made in the presence of A23187 to maximize calcium ATPase activities, and NaN_3 to inhibit mitochondrial ATPase activities. The hibernating preparation had the greatest activity for both ATPases, however the values were not significantly greater than the nonhibernating groups with the exception of the summer group which was significantly lower ($P < 0.05$) than all other groups. Although the magnesium (basal) ATPase activities were variable, they were considerably lower than the calcium ATPase activities. The values for the calcium ATPase activity presented here, using the inorganic phosphate method of analysis, are higher than those reported for rat cardiac SR preparations made using the method of Harigaya and Schwartz (1969) (Ganguly et al., 1983). In contrast, the magnesium ATPase activities reported here are considerably lower than those reported for rat cardiac SR preparations where activities can equal, or exceed, values reported for the calcium ATPase (Penpargkul, 1979; Lopaschuk et al., 1983).

In contrast to the calcium ATPase activities measured using the

Table 2.4 Basal (magnesium) and calcium ATPase activities of cardiac SR preparations as measured using the inorganic Pi colorimetric method at 37°C. The reaction mixture contained 30 μg/ml A23187 to maximize SR calcium ATPase activity. The magnesium ATPase activity was statistically similar for all groups except for the summer group which was significantly lower than the hibernating group ($p < 0.05$, star). The calcium ATPase activity in the summer group was significantly lower ($p < 0.05$) than the other groups (star), which had similar activities. Shown are mean ± standard error.

ATPase ACTIVITIES

	n	Mg-ATPase	Ca-ATPase
HIBERNATING	6	3.85 ± 1.13	28.13 ± 2.83
SPRING	5	1.09 ± 0.43	25.20 ± 1.44
SUMMER	7	0.94 ± 0.48*	16.55 ± 1.45*
FALL	6	2.20 ± 0.82	22.05 ± 1.55
WINTER	6	1.35 ± 0.51	23.49 ± 3.15

(μmol Pi/mg protein/hr)

inorganic phosphate method, the time course of calcium ATPase activity measured using the NADH linked enzyme assay presented in Figure 2.5 did not show an activity profile for the summer group that was significantly lower than the other nonhibernating groups. In addition, the hibernating group was significantly greater than the nonhibernating groups (spring, summer and fall) over the latter time points (minutes 6 and 7). As in the previous assay, A23187 was added to maximize calcium ATPase assay.

The time course of calcium ATPase activities measured in the absence of A23187 at 37°C and at 19°C are illustrated in Figures 2.4 and 2.6 respectively. Although the hibernating group had the highest calcium ATPase activity over time at 37°C (Fig. 2.4) it is not significantly greater than the nonhibernating groups represented here. At 19°C (Fig. 2.6) the calcium ATPase activities for the different groups were decreased in comparison to those obtained at 37°C; the hibernating group however, gave an activity profile that was significantly greater ($p < 0.05$) than the nonhibernating groups after the 3 minute time point.

The initial rates of calcium ATPase activity obtained from the NADH linked enzyme assay are shown in Table 2.5. The hibernating group had the highest initial rate of calcium ATPase activity at 37°C in the presence of A23187 ($p < 0.05$). In the absence of A23187 at 37°C the initial rate of the hibernating group was considerably lower and no longer significantly greater than the nonhibernating groups (p values were however, close to indicating a significant difference between

Figure 2.4 Calcium ATPase activities for hibernating (n = 5), fall (n = 7), summer (n = 6) and spring (n = 5) animals as measured using the NADH enzyme linked assay at 37°C in the absence of A23187. Although the hibernating group had the highest rate over time it was not significantly greater than the other nonhibernating groups at this temperature. Symbols represent mean values, +1 standard error is shown on every second time point.

CALCIUM ATPase ACTIVITY (NADH ASSAY)

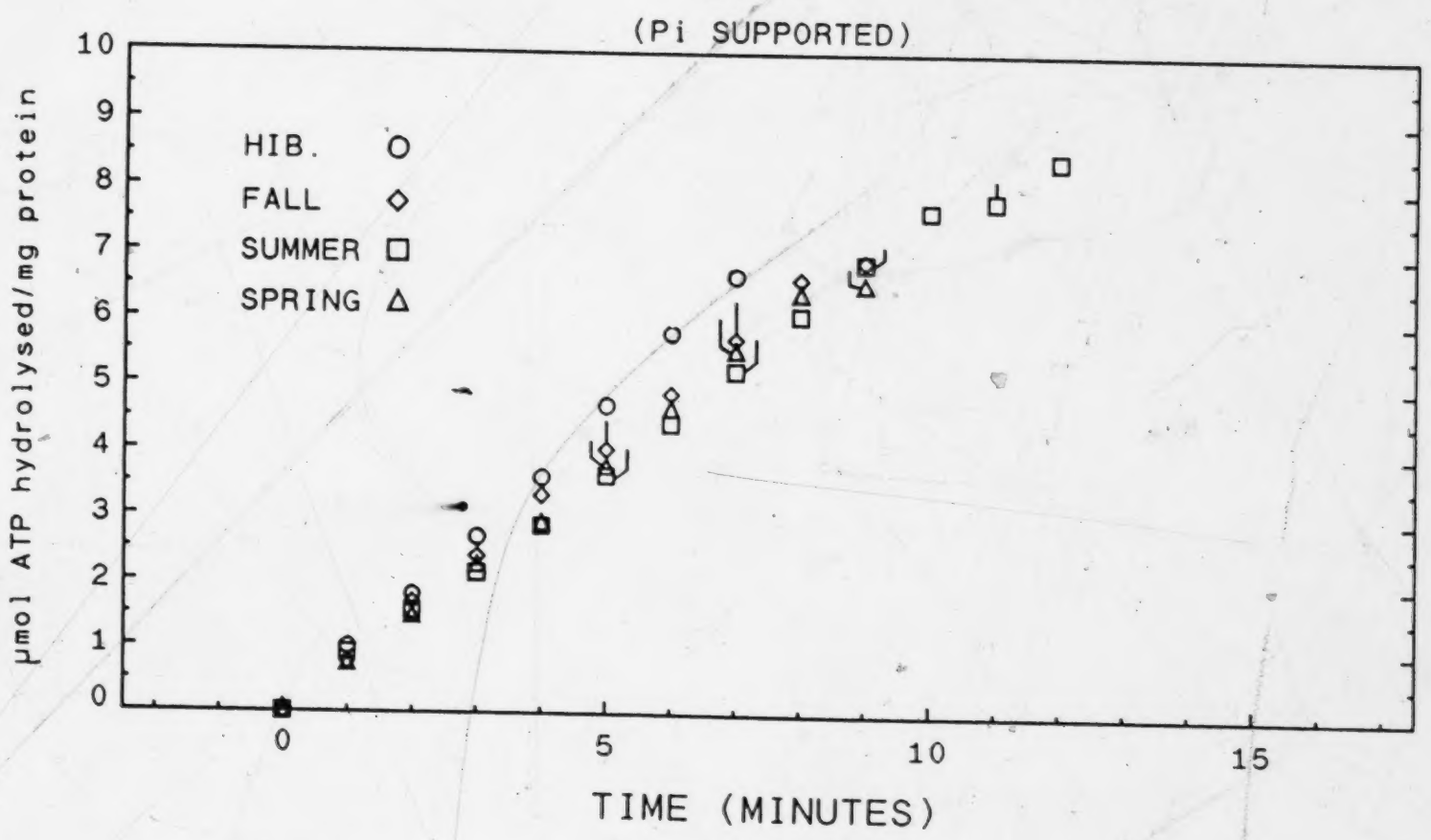


Figure 2.5 Calcium ATPase activities for hibernating (n = 5), fall (n = 7), summer (n = 6) and spring (n = 5) animals as measured using the NADH enzyme linked assay at 37°C in the presence of 30 μg/ml A23187. The calcium ATPase activity of the hibernating group was significantly greater ($p < 0.05$) than the nonhibernating groups for the last two time points (minutes 6 and 7). Symbols represent mean values, +1 standard error is shown on every second time point.

40

CALCIUM ATPase ACTIVITY (NADH ASSAY)

(Pi SUPPORTED, A23187 PRESENT)

37 °C

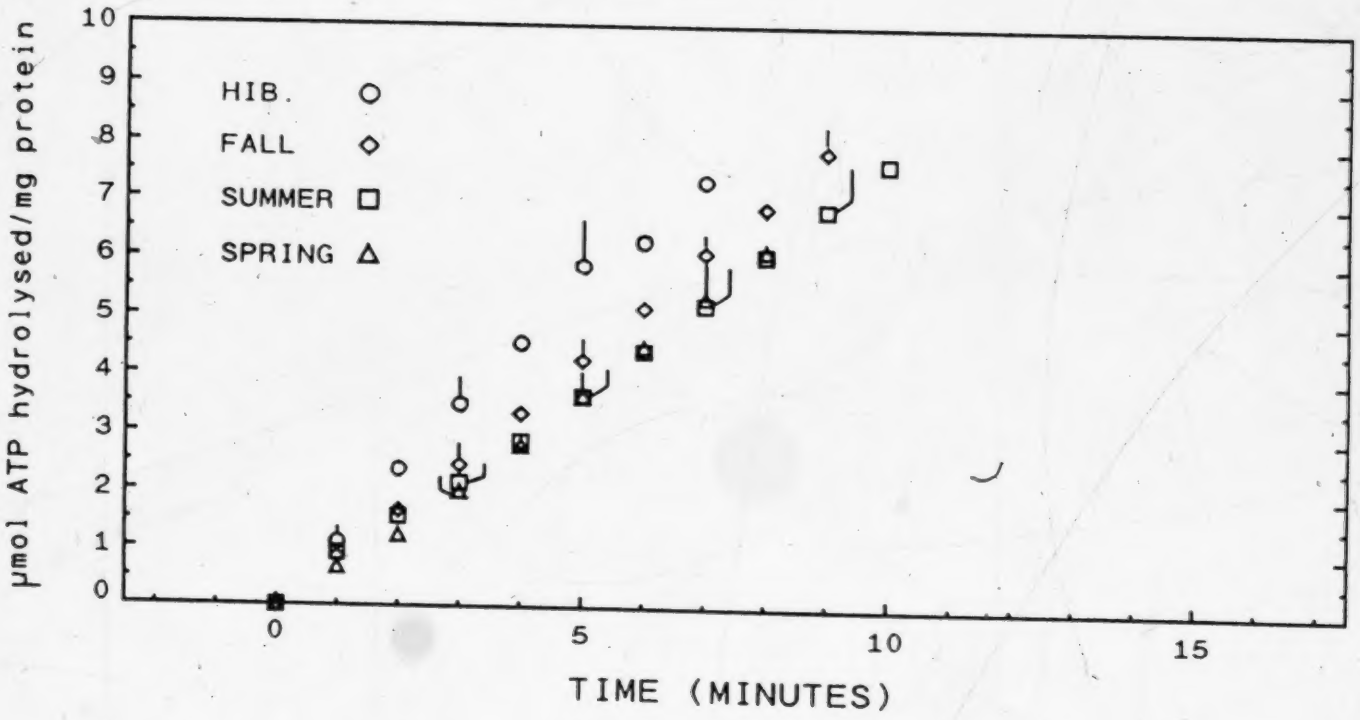


Figure 2.6 Calcium ATPase activities for hibernating (n = 5), fall (n = 7), summer (n = 6) and spring (n = 5) animals as measured using the NADH enzyme linked assay at 19°C in the absence of A23187. The hibernating group showed a significant increase in activity over the other groups ($p < 0.05$) after the 3 minute time point. No significant difference was observed amongst the nonhibernating groups. Symbols represent mean values, +1 standard error is shown on every second time point.

CALCIUM ATPase ACTIVITY (NADH ASSAY)

19 °C

(Pi SUPPORTED)

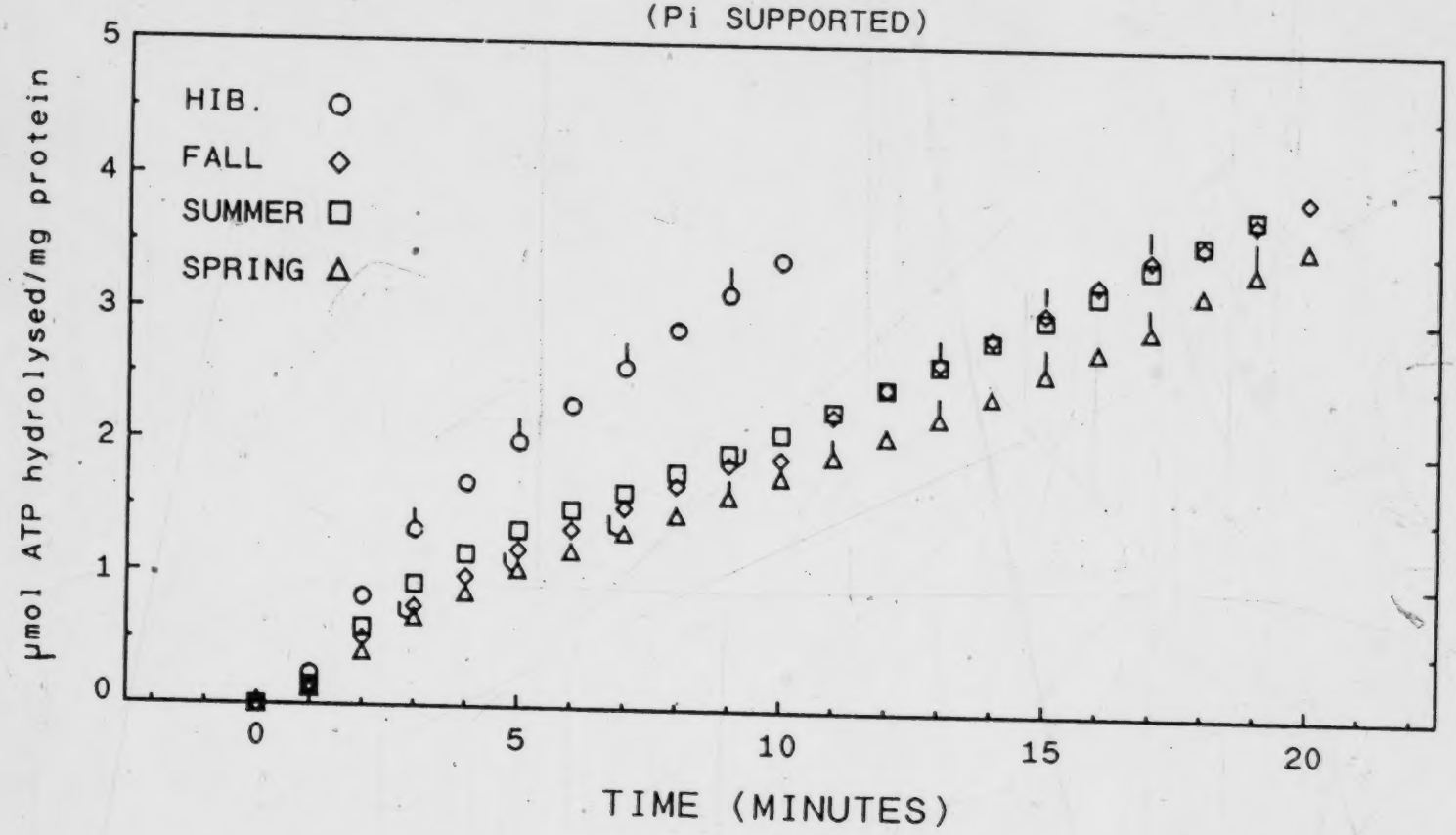


Table 2.5 The initial rates of the calcium ATPase activities for hibernating, spring, summer and fall groups as obtained from the spectrophotometric measurements made using the NADH linked enzyme method. The initial rates were determined by subtracting the initial rate of the reaction in the absence of calcium (ie. in the presence of EGTA) from the initial rate in the presence of calcium. The initial rates calculated from the hibernating group were significantly greater at 19°C (star) in the absence of A23187, and at 37°C (star) in the presence of A23187 ($p < 0.01$ and $p < 0.05$ respectively). Shown is mean ± standard error.

INITIAL RATE OF CALCIUM ATPase

	n	37°C	19°C	37°C + 30 µg/ml A23187
HIBERNATING	5	1.28 ± 0.13	0.56 ± 0.06*	1.62 ± 0.24*
SPRING	6	1.00 ± 0.09	0.30 ± 0.04	0.94 ± 0.18
SUMMER	6	0.88 ± 0.13	0.31 ± 0.01	0.90 ± 0.07
FALL	7	0.97 ± 0.08	0.32 ± 0.03	1.03 ± 0.06

(µmol ATP hydrolysed/min/mg protein)

groups: hibernating vs. spring $p = 0.089$, hibernating vs. summer $p = 0.057$ and hibernating vs. fall $p = 0.059$). The initial rate of calcium ATPase activity demonstrated by the hibernating group at 19°C was significantly higher ($p < 0.01$) than that calculated for the nonhibernating groups.

D. DISCUSSION

One problem in conducting a seasonal study on cardiac SR is the possibility that variations in non-SR protein in the samples will lead to variations in the expressed activities (ie. calcium ATPase and calcium uptake), as all values are normalized on the basis of the amount of protein added to the reaction (Panagia et al., 1985). No significant variations for either the mitochondrial or sarcolemmal enzyme markers was observed among the different groups even though variations in protein yield occurred. In addition, the presence of these contaminants in the SR preparations was relatively low. If however, a significant increase in a protein not of SL or mitochondrial origin occurred, and this was not assayed for, then it would be expected that in those groups showing a higher protein yield the assays involving SR functions would be decreased owing to the dilution effect on the SR protein. Conversely, an undetected decrease in a protein would cause a concentrating effect on the SR activities assayed. In the present study a significantly lower protein yield was observed in the winter group, while the hibernating group had one of the highest protein yields (Figure 2.3). Given the scenario outlined above, the winter group would be expected to have higher SR related activities than the hibernating

group; in fact, the opposite is true, for the basal and calcium ATPase activities presented in Table 2.4 and Figure 2.4, and for the studies on calcium uptake and calcium accumulation presented in Chapter 3. The calcium ATPase activities for the winter and hibernating animals presented in Table 2.4 do not even suggest an increase in contaminating proteins in the hibernating state, as the hibernating group demonstrated the highest level of enzyme activity, even though it was not statistically significant from the winter group. The enzyme marker assays for mitochondria and SL contamination also dispute this idea as the hibernating group demonstrated higher levels of these contaminants (although not significantly higher) in relation to the winter group. The summer and winter groups, which showed the greatest difference in protein yield, were also significantly different from each other with respect to calcium ATPase activities as presented in Table 2.4 indicating a nonSR protein is diluting the SR protein in the summer group thus reducing the measured enzyme activities. This interpretation however, may be incorrect as the decreased calcium ATPase activity of the summer group, in relation to the other nonhibernating groups, seen in Table 2.4 is not demonstrated in the NADH linked enzyme assay (Fig. 2.5). The reason for this difference between the two methods is unknown. Generally however, it may be concluded that while small changes in nonSR proteins probably occurred between the different groups, leading to the minor variations between the groups, it is unlikely that drastic changes in the level of proteins of non-SR origin occurred to account for the results presented in this and subsequent chapters.

The SR preparations from all groups had low basal magnesium ATPase activities in comparison to the measured calcium ATPase activities (Table 2.4). This result differs from that seen in rat where cardiac SR usually shows a high magnesium ATPase activity relative to calcium ATPase activity (Penpargkul et al., 1981; Penpargkul, 1978; Ganguly et al., 1983). In addition, the high magnesium ATPase seen in the hibernating group in Table 2.4 (although not significantly higher than any other group except the summer animals) contradicts the results seen by Charnock et al. (1980) in their SL preparation in which a decrease in magnesium ATPase activity occurred in Richardson's ground squirrels during hibernation. It is difficult to interpret these results as the physiological significance of this ATPase is currently unknown (Charnock et al., 1980; Tate, 1986). It is interesting to note however, that the decreased magnesium ATPase in Charnock et al.'s study is accompanied by a decrease in Na^+-K^+ ATPase activity. Likewise, an earlier study by Charnock and Simonson (1978) on plasma membrane vesicles from hibernating Richardson's ground squirrel brains, revealed that an increase in Na^+-K^+ ATPase was accompanied by an increase in magnesium ATPase. Thus it would seem that for excitable tissues, such as cardiac and neural tissue, the magnesium ATPase activity follows changes in the activity of other enzyme species, in this case the Na^+-K^+ ATPase. In our case, the increased magnesium ATPase in the hibernating state follows the high calcium ATPase activity seen in our SR preparation (Table 2.4 and Fig. 2.5). The observation that the magnesium ATPase activity is highest in SR preparations made from hibernating animals, which contradicts the results seen by Charnock et al. (1980) for their

SL preparation, indicates that plasma membrane contamination in our preparations is low.

The hibernating group had the highest calcium ATPase activity measured in the presence of A23187, at 37°C, using both the inorganic phosphate method (Table 2.4) and the NADH linked enzyme method (time course - Fig. 2.5; initial rates - Table 2.5). A23187, a calcium ionophore, serves to abolish any calcium retention within the SR vesicles by making them leaky and thus preventing a feedback inhibition on the calcium ATPase by calcium (Madeira, 1982). This uncoupling of calcium accumulation and calcium ATPase allow us to view the intrinsic rate of the calcium ATPase by allowing the enzyme to cycle freely. The hibernating preparations, therefore, have the highest intrinsic rates of calcium ATPase activity. The reason for this high rate is unknown, as these results can occur either by increasing the levels of this enzyme in the preparation, or by phosphorylation of phospholamban which increases the cycling rate of the enzyme by decreasing the time spent in the phosphorylated state (Shamoo and Ambudkar, 1984).

The calcium ATPase activity measured in the absence of A23187 represents the functional enzyme activity when coupled to calcium accumulation. Both the time course (Figures 2.4 and 2.6) and initial rate data (Table 2.5) indicate that the hibernating group has the highest rate of calcium ATPase (although not significantly higher at 37°C), and that this rate is less sensitive to temperature change than that seen in the nonhibernating groups (spring, summer and fall) when the temperature is decreased from 37 to 19°C. Overall, the high calcium ATPase in the hibernating group suggests, baring a low Ca^{2+}/ATP coupling

ratio, that this group should have the highest rate of calcium uptake across most of the physiological Tb range encountered. This is indeed true (see Chapter 3). As to why the thermal sensitivity of this enzyme is lowest during the hibernating phase is currently unknown. It is possible that a seasonal change in some physical parameter such as membrane lipid composition or calcium ATPase protein conformational state could be involved. Such changes could lead to a change in the activation energy, or a lowering, or elimination, of the break point (around 21-20°C) seen in Arrhenius plots of SR calcium ATPase in rats and other species (de Meis, 1981; Abeywardena et al., 1984). Changes from non-linear to linear Arrhenius plots of membrane bound enzymes commensurate with hibernation have been noted previously in hamsters (Houslay and Palmer, 1978) and Richardson's ground squirrels (Pehowich and Wang, 1984). In the latter study the break point of 21°C for mitochondrial succinate dehydrogenase found in summer animals disappeared entirely in the hibernating preparations when measured down to 4°C and this change is not due to the downward shift of the lipid phase transition temperature (Pehowich et al., 1988). It is possible that some yet unknown physical parameters in the SR membrane are altered during hibernation to make the hibernating SR calcium ATPase less sensitive to a decrease in temperature in relation to the spring, summer and fall groups.

Although the hibernating group clearly has the highest calcium and magnesium ATPase activities in the presence and absence of A23187 (although not significantly higher in all cases), analysis of the data from spring, summer and fall groups does not result in an easily

definable seasonal trend. In the inorganic phosphate assay (Table 2.4) the fall group had the second highest magnesium ATPase activity (although not significantly different from the other groups), and in the NADH linked enzyme assay, the time course of the calcium ATPase at 37°C, in the presence and absence of A23187, was greater than the spring and summer groups (although again, not significantly greater). This result was however, not observed when the NADH linked enzyme assay was conducted at 19°C (Fig. 2.6). The spring group while having the second highest calcium ATPase in the inorganic phosphate assay (Table 2.5), failed to show this trend in the NADH linked enzyme assay (Figure 2.5). The summer animals had the lowest activity in all assays at 37°C. Thus, apart from the hibernating group giving the highest values in all enzyme assays, a clear seasonal transition in calcium ATPase activity is not readily apparent.

Becker and Willis (1974) failed to demonstrate a cold resistance of skeletal muscle SR calcium ATPase isolated from a 13-lined ground squirrel and a rat, they noted that this may be due to the relative inactivity of skeletal muscle during hibernation, and suggested a more active muscle might provide different results. Although this study was not conducted between a hibernating and nonhibernating species, changes in calcium ATPase activity do occur between the nonhibernating and hibernating states in the Richardson's ground squirrel, resulting in an increase of the intrinsic level of activity, and a decrease in the thermal sensitivity of the enzyme in the hibernating state.

As the SR calcium ATPase activity is intimately related to the removal of calcium from the cytoplasm to cause relaxation, its activity

has been positively correlated with the ability of cardiac muscle to relax. In cardiac myopathies, such as that caused by diabetes, a decrease in calcium ATPase activity, and consequently calcium uptake, are reflected in the decreased ability of the cardiac muscle to relax after contraction (Ganguly et al., 1983). In accordance with the data presented above, and the data presented in the next chapter, the myocardium of hibernating Richardson's ground squirrels shows a significantly greater rate of relaxation than that of animals in the nonhibernating state (Zhou et al., unpublished observations and data included in Appendix A at the end of this thesis). It is interesting to note that the absolute rate at which the muscle relaxes is similar at 36°C (-dF/dT: hibernating = 8.4 mN/s/mg tissue, nonhibernating = 7.2 mN/s/mg tissue), but increases in the hibernating group as temperature decreases. The results presented here are in agreement with the general notion that calcium ATPase activity is indicative of myocardial performance, especially myocardial relaxation. The increased calcium ATPase activity in the hibernating group at 19°C reflects the increased capability during hibernation to deal with any possible increase in the reliance of internal calcium cycling during hibernation (Zhou et al. unpublished observations, see the general introduction).

In summary, contamination of the SR preparations by mitochondrial and sarcolemmal membranes was low, and seasonally, they did not vary significantly under different physiological states. While variations in SR protein yield was noted between the different groups these do not appear to represent changes in nonSR protein because such a change could not account for the results observed herein. The high calcium ATPase

activity in the hibernating group, in both the coupled and uncoupled states, provides a cellular mechanism for the increased rate of relaxation seen in the hibernating myocardium by allowing SR to accumulate calcium at a faster rate, especially at lower Tb during hibernation.

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III. SEASONAL VARIATIONS IN SR CALCIUM UPTAKE AND CALCIUM ACCUMULATION.

A. Introduction

Sequestration of calcium by SR via the calcium ATPase pump is important in the relaxation of cardiac muscle. SR can accumulate calcium against a large concentration gradient (approximately 1 mM inside verses 10^{-7} M outside)(Blinks, 1986). The stored calcium is released from the junctional SR for the subsequent contraction. Therefore, calcium accumulation by SR is important in determining the mechanical properties of the muscle, as it determines the amount of calcium available for contraction. In addition, the calcium accumulating function of the SR is important in establishing a low intracellular calcium concentration (Carafoli, 1985). In animals which undergo changes in body temperature, calcium release and reaccumulation must occur at all temperatures over which the animal survives. In hibernating animals this must occur between 37°C and 1-2°C (Lyman and Blinks, 1959).

Early studies on the effects of temperature on cardiac SR calcium uptake shows a linear relationship for the Arrhenius plots (Inesi and Watenabi, 1967; Chiesi, 1979); however, studies incorporating more temperature points indicate a break point at 20°C (Berman and Aderen, 1981) which is more consistent with observations seen in skeletal SR. It is unknown how decreasing temperature affects calcium uptake by cardiac SR in a hibernating species, within either the hibernating or nonhibernating phase. Increased reliance upon internally stored calcium for contraction in hibernators has been suggested by Kondo and Shibata (1984, see General Introduction), although it is unknown what changes

may occur, if any, in the SR to facilitate the uptake of calcium, and how this may be affected by the low temperatures at which the hibernators must survive. This study examines calcium uptake and accumulation by cardiac SR from hibernating and nonhibernating Richardson's ground squirrels. Included are both seasonal aspects and the influence of temperature on SR activity.

The objectives of this study are: a) to determine the initial rates of calcium uptake by cardiac SR preparations from the different groups, b) to examine the amount of calcium which the different SR preparations can accumulate, and c) to determine the amount of SR calcium accumulation stimulated by ryanodine, a compound which blocks calcium release from junctional SR (Besch, 1985). Experiment b) documents the function of the nonjunctional SR in the preparation, while experiment c) documents the function of the junctional SR in the preparations.

B. Materials and Methods

Animals and SR Preparation

The animals used in this experiment, and the method for the isolation of the cardiac SR microsomal fractions, were the same as those used in Chapter 2.

The Calcium Electrode Method

Calcium uptake by SR vesicles was monitored continuously using a calcium electrode (Ca^{2+}-Selectrode, F2112 Radiometer) (Madeira, 1975). The uptake of calcium by SR was measured in a temperature controlled

vessel (37-4°C), at 3°C intervals, using 2 ml of the same reaction medium used in the inorganic phosphate method of measuring calcium ATPase activity described in Chapter 2. The pH of the solution was continuously monitored by a PHM 64 research pH meter (Radiometer), and adjusted to pH 7.38 initially (at all temperatures) by the addition of KOH (200 nmol/2 µl added). A total of 100 nmol of $CaCl_2$ in 10 µl was added to the reaction medium, with 80 nmol (8 µl) being added initially, and the final 20 nmol (2 µl) addition being used to calibrate the calcium electrode. After equilibration of the electrodes, calcium uptake was started by the addition of 50 ul of SR suspension to the reaction medium (typically 30-100 µg of protein/ml of reaction medium). Changes in pH and Ca^{2+} concentration of the reaction mixture were recorded simultaneously using a dual pen chart recorder. After completion of the experiment a 2 ml aliquot of solution was removed for analysis of protein concentration as described in Chapter 2.

The initial rates of calcium uptake were determined by drawing a tangent to the steepest portion of the recorder tracing and then calculating the length of time needed to accumulate 20 nmol of calcium (see Madeira, 1975). Results were then calculated as the nmols of calcium accumulated/minute/mg protein, and expressed as an Arrhenius plot to compare the thermal sensitivities of the different seasonal groups.

The $^{45}Ca^{2+}$ Method

The rate and extent of $^{45}Ca^{2+}$ uptake was also measured using the millipore filtration method (Martinosi and Feretos, 1964). The

experiment was conducted in a manner similar to that described for the calcium electrode, with some modifications. The experiment used 6 ml of reaction mixture, and the pH of the solution was set at 7.38 (for all temperatures) before the addition of 60 µl of $^{45}Ca^{2+}$ (300 nmol $^{45}CaCl_2$, 5,000-2,500 CPM/nmol). After filtering two 0.4 ml aliquots through 0.45 um filters (blanks), $^{45}Ca^{2+}$ uptake was initiated by the addition of 65 ug of SR protein. Aliquots (0.4 ml) were removed and filtered at various times, with each filter being washed twice with 3 ml of a release inhibiting medium containing 20 mM tris maleate (pH 7.38, at 25°C), 100 mM KCl, 100 mM sucrose, 10 mM $MgCl_2$, 5 mM NaN_3 and 10 µM ruthenium red, a SR calcium release blocker (Chamberlain et al., 1984; Besch, 1985) .

The amount of $^{45}Ca^{2+}$ trapped by the vesicles was determined by placing the filters in 7 ml of a scintillation cocktail comprised of (for 4 L volume) 2.76 L of toluene (scintillation grade, Fischer), 1.24 L triton X-100 (Terochem), and 20 g of Omnifluor (New England Nuclear). Samples were counted for two minutes in a liquid scintillation counter (LKB Wallac, model 1714).

$^{45}Ca^{2+}$ Accumulation

The extent of SR $^{45}Ca^{2+}$ accumulation was determined by allowing the experiment described above to continue until the amount of $^{45}Ca^{2+}$ trapped within the vesicles stabilized over time. Under identical conditions the drug ryanodine (0.5 mM) was added to the reaction mixture to increase calcium storage in the junctional SR to determine the amount of this type of SR present in the preparation. This concentration of

ryanodine has previously been shown to the most effective in blocking calcium release from junctional SR (Jones and Cala, 1981).

Statistical analysis of data was performed for each experimental temperature or time point by using an unpaired t-test to determine if groups were statistically different from each other ($p < 0.05$).

C. Results

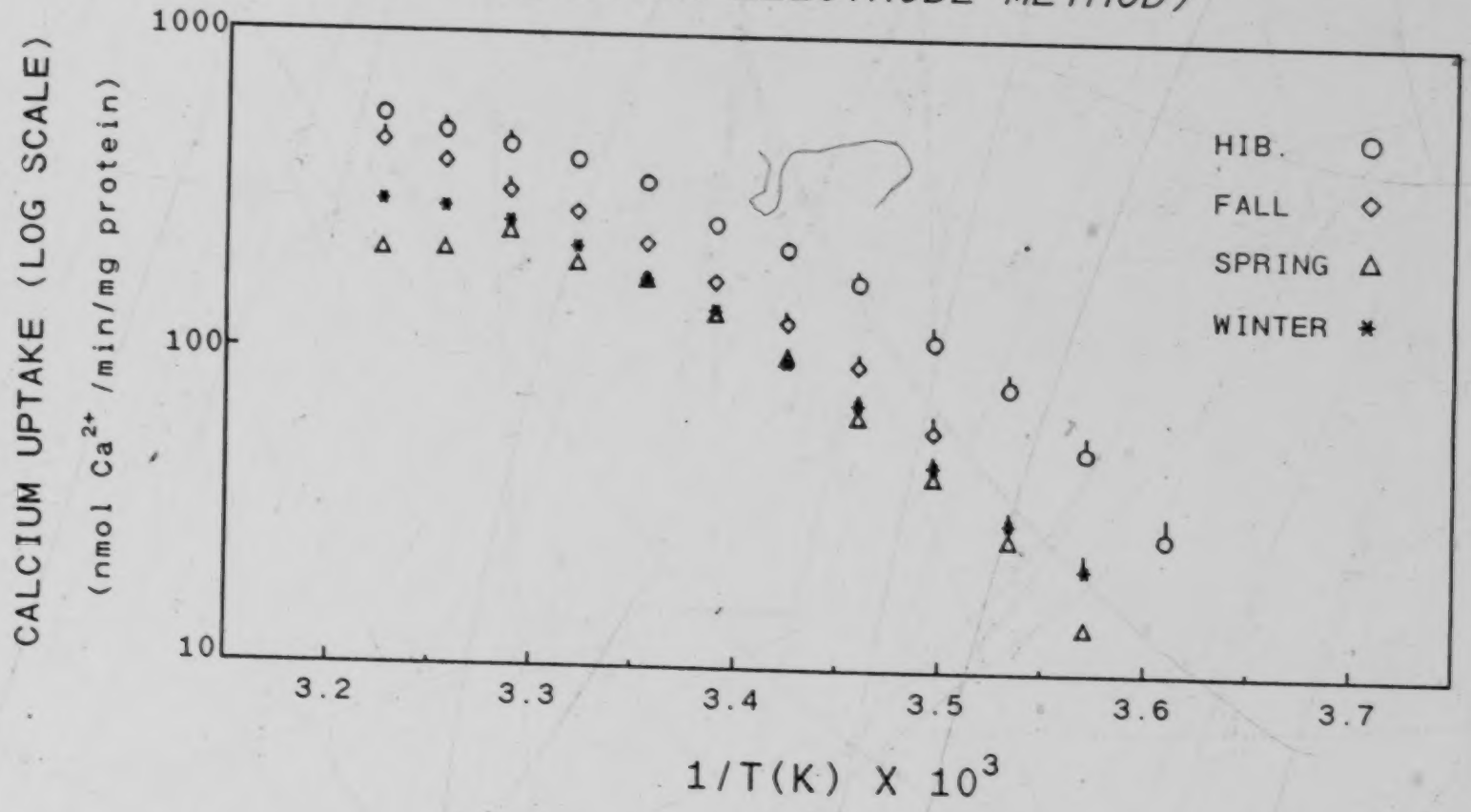
The Calcium Electrode Method

Seasonal changes in the initial rate of calcium uptake at different temperatures, as measured using the calcium electrode are presented in Figure 3.1. The Arrhenius plots were curvilinear, showing no clearly defined breakpoint, making a determination of slopes, and therefore energy of activation, difficult. The initial rates of calcium uptake in the hibernating squirrels were higher than any other group over the temperature range studied. The winter and spring groups showed the lowest rates of calcium uptake, with the two groups being almost superimposable. Although calcium uptake in the fall group was only studied at temperatures between 37 and 13°C, the initial rates of calcium uptake were higher than in the winter group, but lower than the hibernating group. Statistical analysis indicated that (1) calcium uptake in the hibernating group was significantly higher ($p < 0.01$) than the spring and winter animals over the entire temperature range studied; (2) calcium uptake in the hibernating group was significantly higher ($p < 0.05$) than that of the fall group at all temperatures studied except

Figure 3.1 Arrhenius plot of the initial rates of calcium uptake for hibernating (n = 5), winter (n = 6 except for 13 and 7°C where n = 5), spring (n = 5) and fall (n = 5) groups as measured by the calcium electrode method. Hibernating animals had higher initial rates of calcium uptake over the entire temperature range of 37-4°C. The spring and winter groups had the lowest initial rates of calcium uptake, being almost superimposable upon each other. The fall groups shows a range of values between the hibernating and the spring/winter groups. Symbols represent mean values, lines represent +1 standard error.

INITIAL RATE OF CALCIUM UPTAKE

(CALCIUM ELECTRODE METHOD)



at 37°C; (3) fall animals had a significantly greater initial rate of calcium uptake ($P < 0.05$) than the winter animals between 37-16°C but not at 13°C.

The $^{45}Ca^{2+}$ Method

The initial rate of calcium uptake, calculated from the data presented in Figures 3.3 - 3.6 using the $^{45}Ca^{2+}$ method, is shown in Figure 3.2. The hibernating group, as in the previous experiment, had the highest rates of calcium uptake, while the other groups, including the fall animals, show the lowest rates of calcium uptake over the temperature range. Calcium uptake in all groups decreases rapidly with decreasing temperature. Too few temperature points exist within this study to make an accurate determination as to the linearity or nonlinearity of the Arrhenius plot.

Calcium accumulation was measured by allowing $^{45}Ca^{2+}$ uptake to continue for 30 min at 37 and 28°C, 45 min at 19°C, and 60 min at 10°C (Figures 3.3, 3.4, 3.5 and 3.6). At 37°C the rate of $^{45}Ca^{2+}$ uptake was initially rapid during the first two minutes, but decreased sharply after this time point, with the amount of calcium accumulated levelling off, or increasing at a much slower rate after the initial two minutes. At lower temperatures similar results were seen, although the reduction in the rate of calcium uptake was more gradual and took longer to occur. At 10°C none of the groups studied reached a plateau over the 60 minutes of the experiment. The level of calcium accumulated by the hibernators was significantly greater than that of any other group studied at all

Figure 3.2 Effect of temperature on the rate of $^{45}Ca^{2+}$ uptake. Points represent the amount of calcium accumulated in the SR vesicles at 1 min of incubation at all temperatures except for 10°C where vesicles were allowed to accumulate calcium for 2 min. The hibernating group had the highest rates of calcium uptake. Symbols represent mean values, lines represent +1 standard error.

ARRHENIUS PLOT OF ^{45}Ca DATA

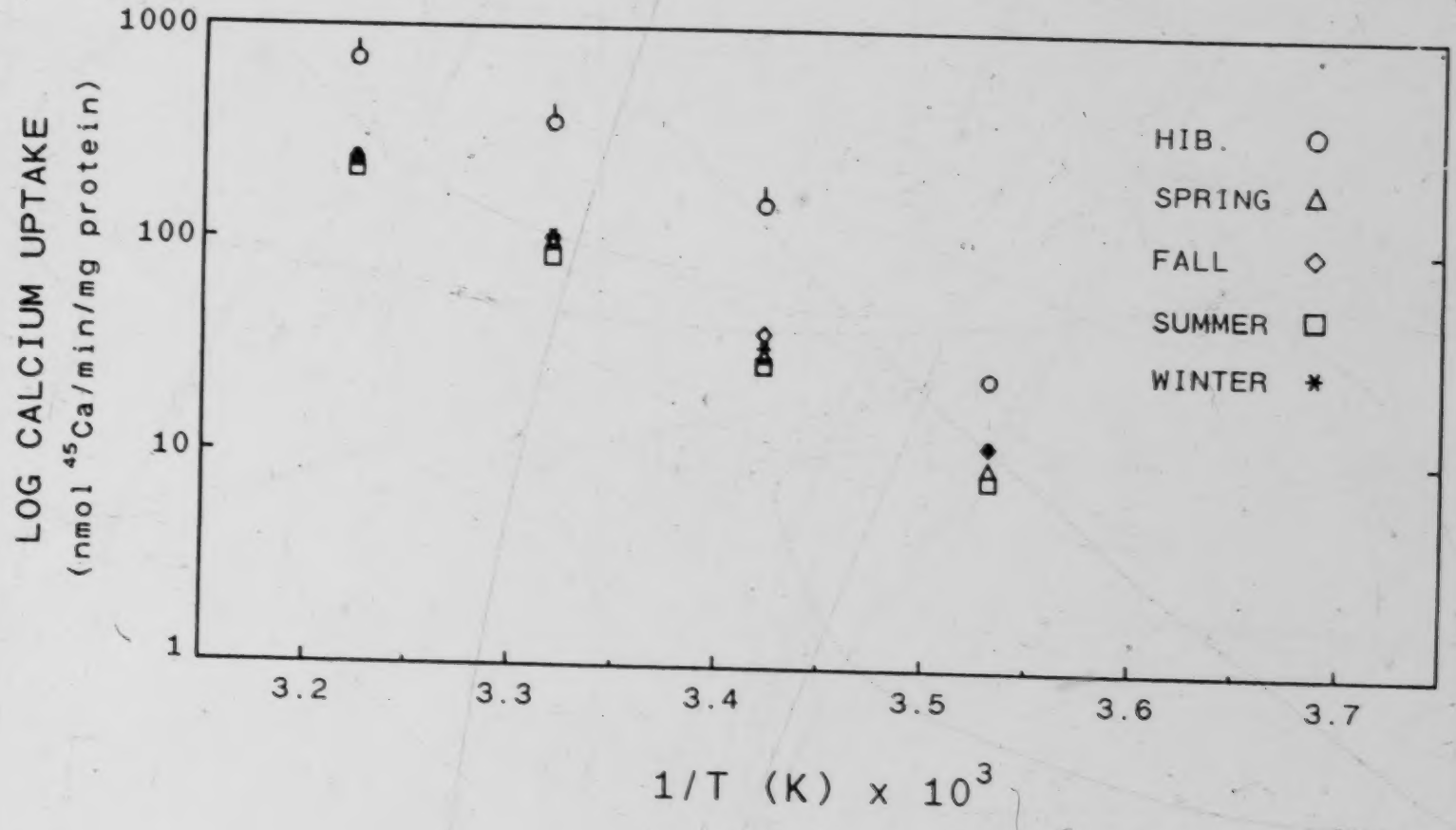


Figure 3.3 $^{45}Ca^{2+}$ accumulation over time at 37°C. Symbols represent mean values, lines represent +1 standard error. The hibernating group had the highest rate of calcium uptake and the greatest extent of calcium accumulation. The summer and spring groups were similar over the time of the experiment, however the fall group showed a greater level of calcium accumulation ($p < 0.05$) over the summer and spring groups at the 20 and 30 minute time points.

CALCIUM(45) ACCUMULATION OVER TIME
(OXALATE SUPPORTED, 37 °C)

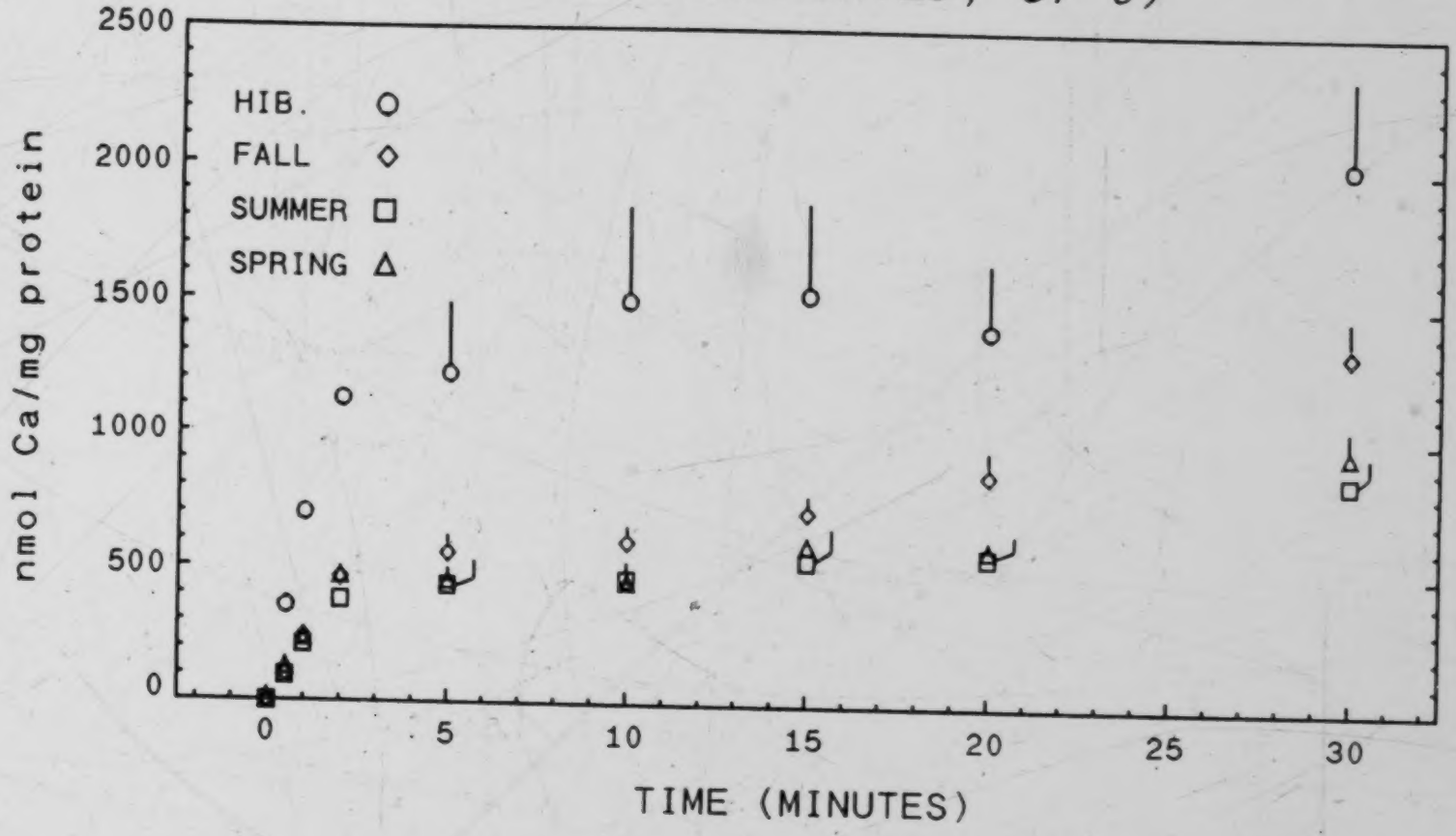


Figure 3.4 $^{45}Ca^{2+}$ accumulation over time at 28°C. Symbols represent mean values, lines represent +1 standard error. The hibernating group had the highest rate of calcium uptake and the greatest extent of calcium accumulation. The fall group demonstrated a greater level of calcium accumulation ($p < 0.05$) over the spring group at the 30 minute time point, and over the summer group at the 15, 20 and 30 minute time points.

CALCIUM(45) ACCUMULATION OVER TIME
(OXALATE SUPPORTED, 28 °C)

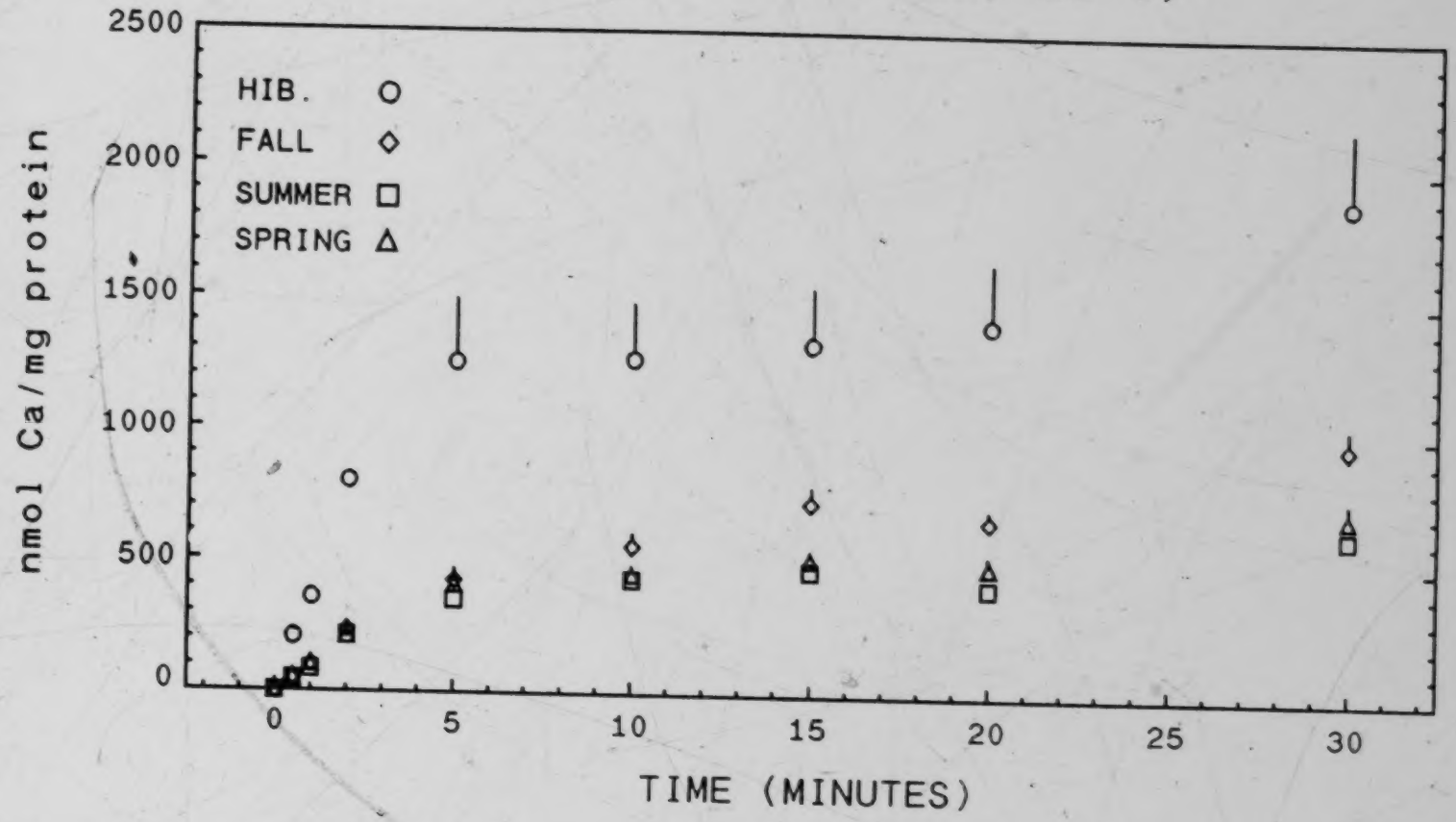


Figure 3.5 $^{45}Ca^{2+}$ accumulation over time at 19°C. Symbols represent mean values, lines represent +1 standard error. The hibernating group had the highest rate of calcium uptake and the greatest extent of calcium accumulation. The fall group demonstrated a significantly increased level of calcium accumulation ($p < 0.05$) in comparison to the summer group at the 30 and 45 minute time points.

CALCIUM(45) ACCUMULATION OVER TIME
(OXALATE SUPPORTED, 19 °C)

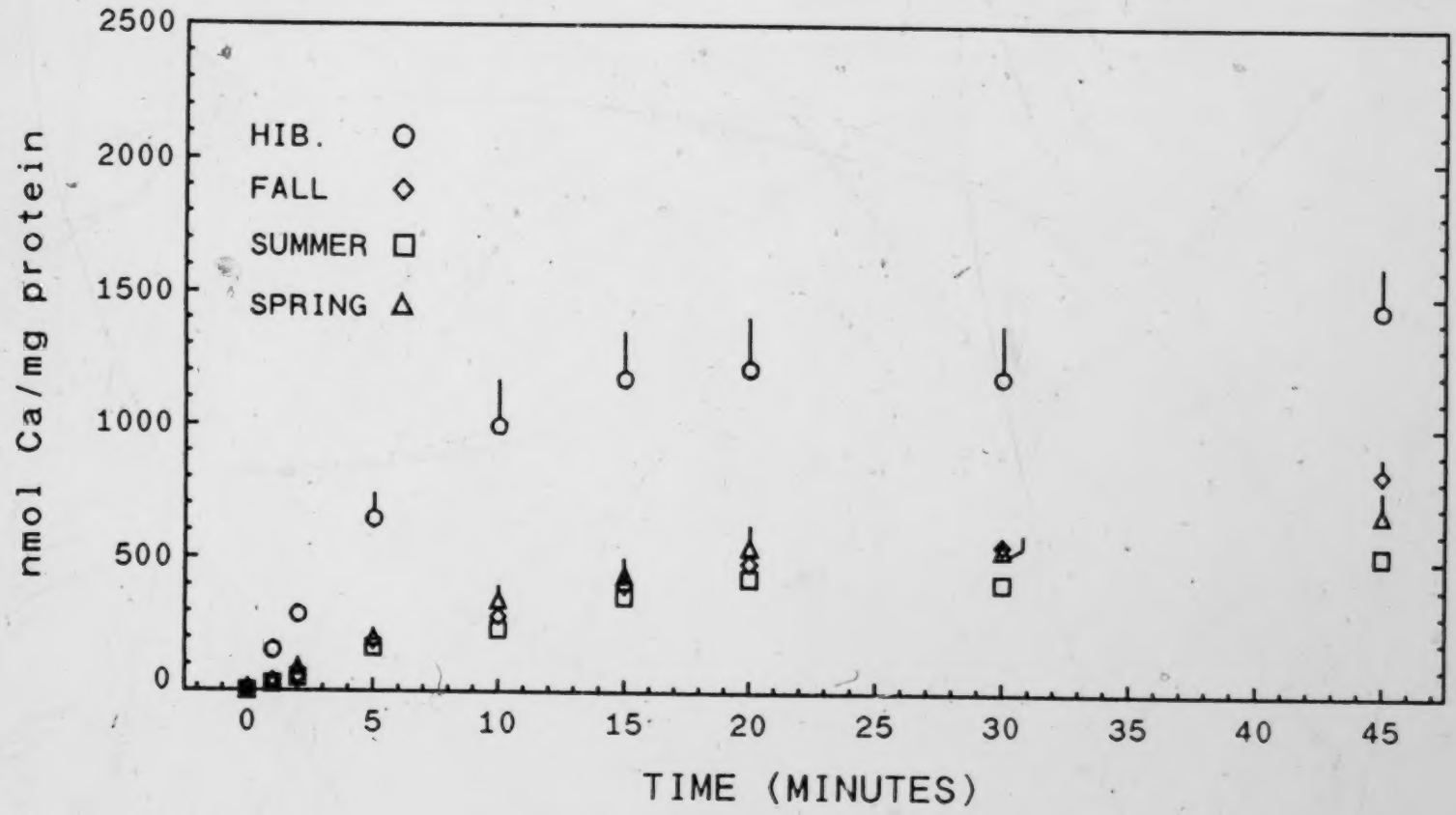
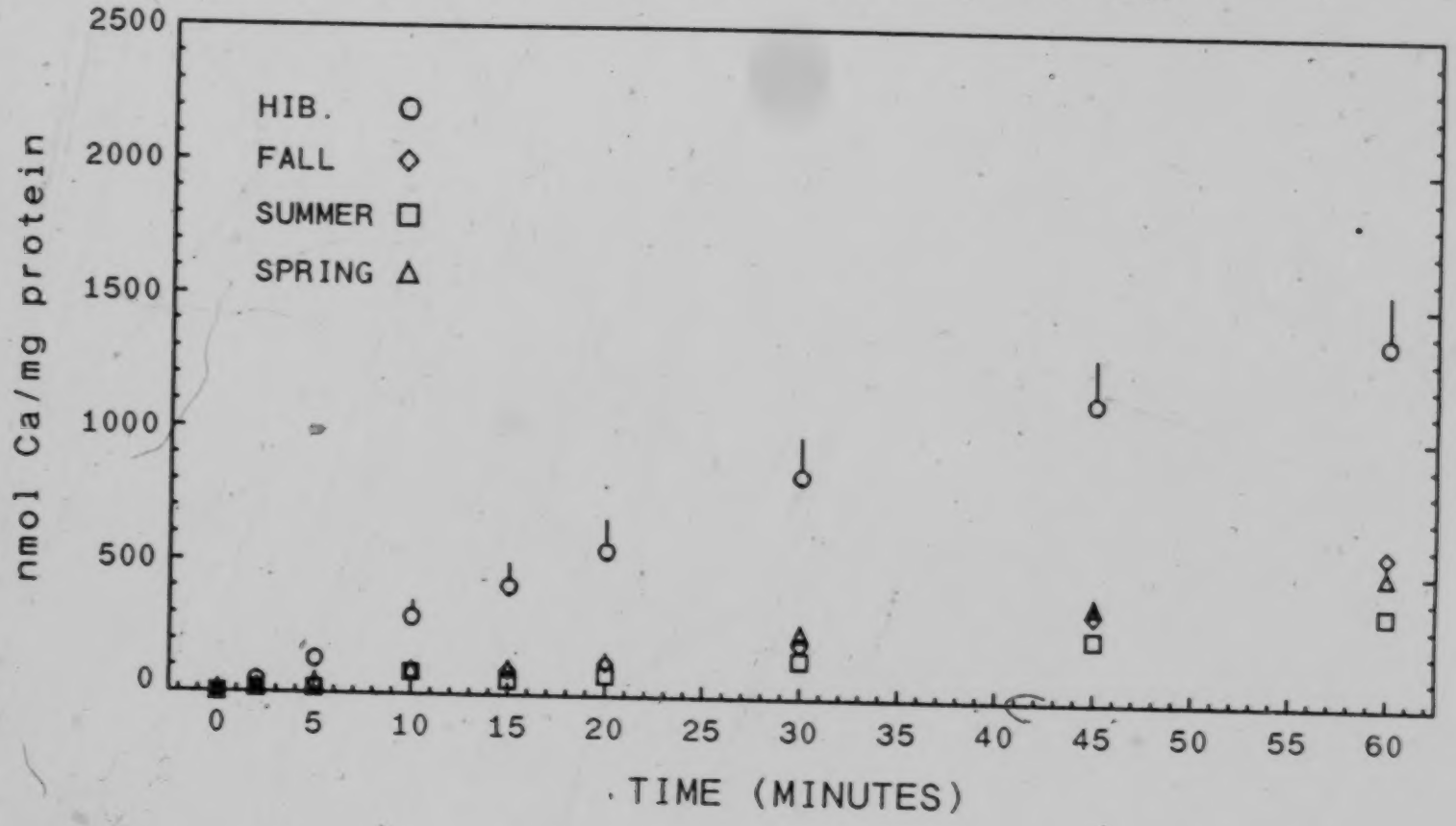


Figure 3.6 $^{45}Ca^{2+}$ accumulation over time at 10°C. Symbols represent mean values, lines represent +1 standard error. The hibernating group had the highest rate of calcium uptake and the greatest extent of calcium accumulation. The fall group demonstrated a significantly increased level of calcium accumulation ($p < 0.05$) in comparison to the summer group at the 60 minute time point.

CALCIUM(45) ACCUMULATION OVER TIME
(OXALATE SUPPORTED, 10 °C)



temperatures. The $^{45}Ca^{2+}$ accumulation plateaus between 1,200 and 1,500 nmol $^{45}Ca^{2+}$ accumulated/mg protein at 37, 28 and 19°C for the hibernating group, while the other groups start plateauing at around 500 nmol $^{45}Ca^{2+}$ accumulated/mg protein or less for the same temperatures. Although the fall animals did not show a significantly greater initial rate of calcium uptake using the $^{45}Ca^{2+}$ method, than they did using the calcium electrode, they did show significantly increased levels of calcium accumulation ($p < 0.05$) over spring animals at 37°C (20 and 30 minute time points) and 28°C (30 minute time point), and over the summer animals at 37°C (20 and 30 minute time points), 28°C (15,20 and 30 min time points), 19°C (45 minute time point) and 10°C (60 minute time point).

The addition of ryanodine to the $^{45}Ca^{2+}$ uptake reaction medium caused an increase in the retention of $^{45}Ca^{2+}$ in all groups studied. Although not statistically significant, the hibernating group had the lowest % increase in ryanodine-stimulated calcium uptake in comparison to the amount of calcium taken up in the absence of ryanodine (Figure 3.7). Comparing the absolute amount of ryanodine-stimulated calcium uptake there was no difference between the groups (Figure 3.8). This Figure also shows the amount of ryanodine stimulated calcium accumulation in relation to the amount accumulated in the absence of ryanodine.

D. Discussion

The SR preparations from animals in the hibernating state

Figure 3.7 The percent increase of calcium accumulation by SR vesicles at 37°C after 30 minute of incubation in the presence of 0.5 mM ryanodine over that in the absence of ryanodine. Although not statistically significant the hibernating group had the lowest mean percentage increase among all groups. Bars represent mean and line represents standard error. n = 6 for all groups except fall where n = 7.

% INCREASE IN CALCIUM ACCUMULATION
(EFFECT OF RYANODINE)

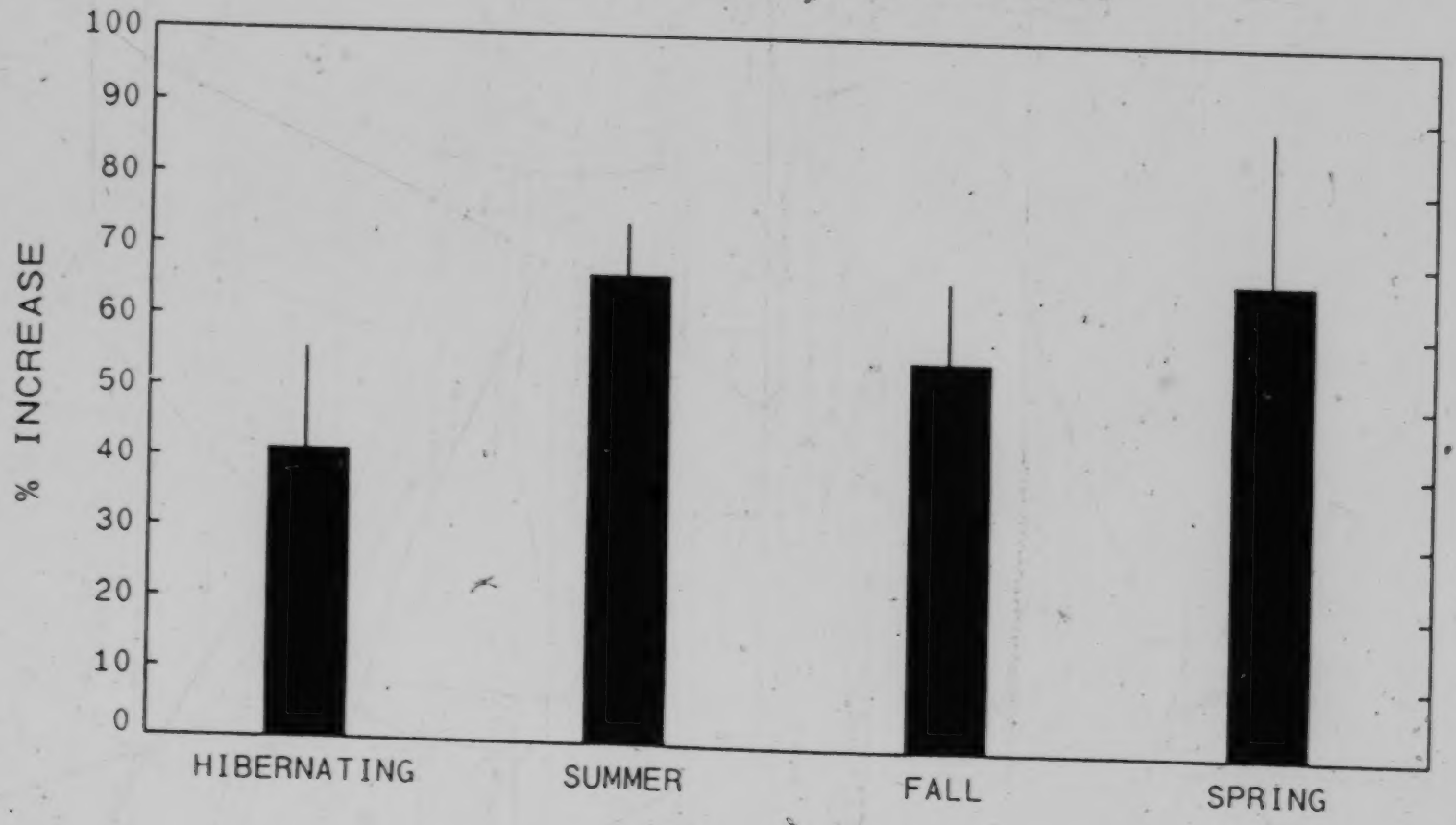
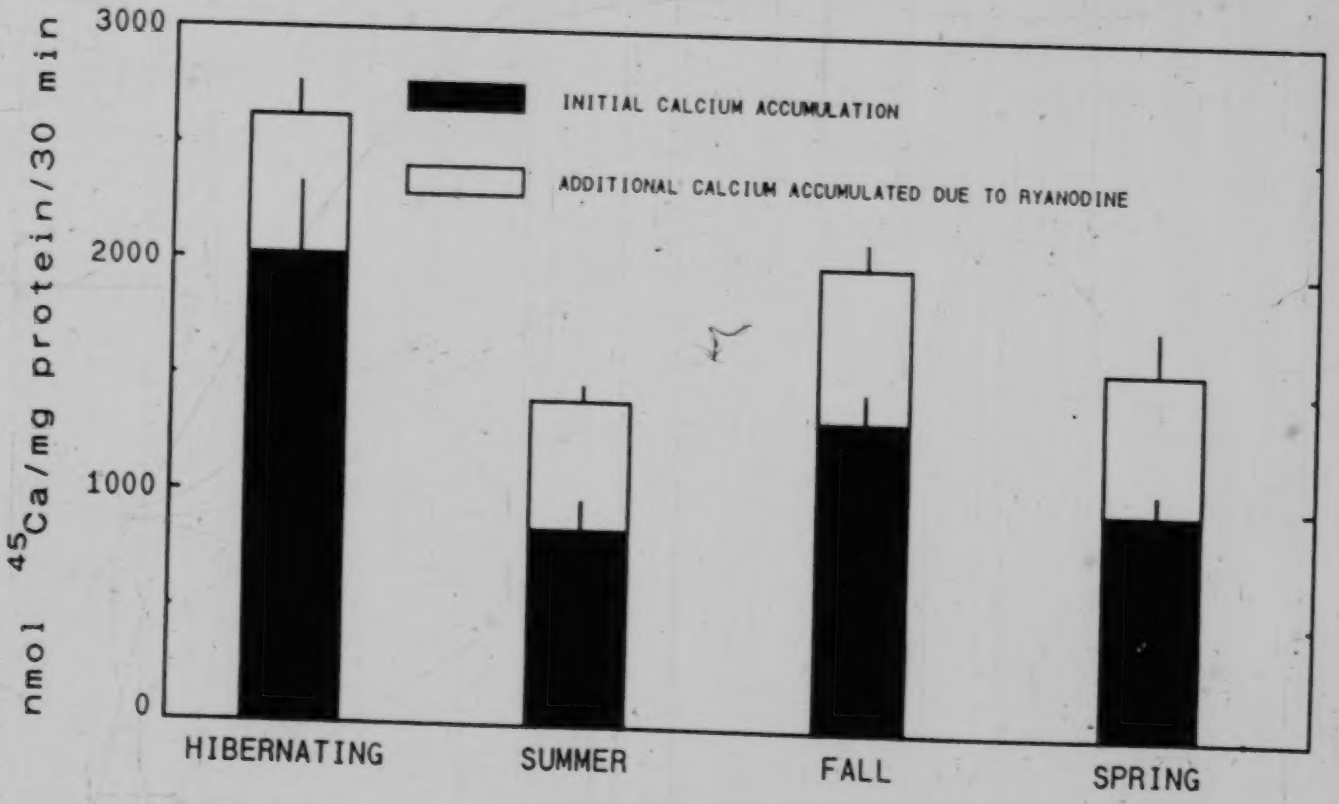


Figure 3.8 The extent to which 0.5 mM ryanodine increased the amount of calcium accumulated by the SR vesicles at 37°C after 30 minute of incubation is demonstrated by the open bars, while the extent of calcium uptake in the absence of ryanodine is demonstrated by the solid bars. Note that the absolute extent to which ryanodine stimulated calcium uptake was similar for all groups ranging from 600-800 nmol calcium/mg protein, despite variations in the levels of calcium accumulation in the absence of ryanodine. n = 6 for all groups except fall where n = 7.

INCREASE IN ^{45}Ca ACCUMULATION DUE TO RYANODINE IN COMPARISON TO INITIAL ^{45}Ca ACCUMULATION



demonstrated the greatest rates of calcium uptake based on both the calcium electrode and the $^{45}Ca^{2+}$ methods. This result was seen at all temperatures studied and not just at the lower temperature as seen for the calcium ATPase study (see Chapter 2). Our study of the initial rate of SR calcium uptake with the calcium electrode agrees with the results of Berman and Aderen (1981), in that the Arrhenius plot is not linear as was reported previously by Inesi and Watenabi (1967) and Chiesi (1979); unlike Berman and Aderen however, the plot was curvilinear making an accurate determination of a break point and activation energies difficult. The increased rate of calcium uptake is important to the maintenance of cellular calcium concentrations as SR is thought to be the most important organelle in reducing and regulating intracellular calcium levels at rest under normal conditions (Carafoli 1985). Unlike the red blood cell study, between guinea pig and hibernator RBCs (see Wang and Wolowyk, 1988 for review), in which the hibernators were able to keep intracellular calcium low, cardiac muscle tissue must have transiently elevated levels of intracellular calcium to function. The ability of the SR to reaccumulate calcium quickly is important to ensure that resting cytosolic calcium concentrations remain at physiologically safe levels, as excess calcium is known to elicit deleterious effects, leading to a loss of cardiac function (Katz and Reuter, 1979, Dhalla et al., 1982).

The reason for the increased SR calcium uptake rate seen in the hibernating state is likely due to either: A) an increase in the amount of SR in the preparation, or B) a modification of the SR to increase its rate of calcium uptake, such as the phosphorylation of phospholamban by

cAMP and calmodulin. Although the increased capacity for calcium accumulation in the hibernating group would seem to indicate the presence of more SR in the preparation, this result has been observed when vesicles are incubated with cAMP or calmodulin along with the appropriate kinases to phosphorylate phospholamban. Several researchers have noted an increase in both the rate and extent of calcium accumulation, in both the presence and absence of precipitating anions (ie. oxalate), by isolated cardiac SR vesicles stimulated with both cAMP and calmodulin (Entman et al., 1969; Fedelesova and Ziegelhoffer, 1975; Kim et al., 1987). The reason for this increase in calcium accumulation by the SR in this case is unknown, but clearly cannot be due to biosynthesis of SR in isolated vesicles; therefore the cAMP is acting upon SR already present in the preparation to increase the levels of calcium accumulation. These results have also been noted in vivo after the application of compounds which increase intracellular levels of cAMP are added to the tissue. By perfusing rat hearts with $^{45}Ca^{2+}$ to label intracellular stores, and then perfusing with cold calcium to stimulate $^{45}Ca^{2+}$ release from these stores, Hunter et al. (1983) found that isoproterenol increases the amount of calcium stored within two intracellular pools, thought to be SR. The reason for the increased calcium accumulation in this case is unknown, however, the increase in calcium accumulation occurs too quickly to be attributed to SR membrane biosynthesis. Thus, although the increased level of calcium accumulation seen in the hibernating state would suggest the presence of a greater amount of SR in the preparation, the possibility that these results represent modification of existing SR though the phosphorylation

of phospholamban cannot be eliminated. It is clear however, that either intervention will result in an increased rate of calcium uptake by SR.

At 37°C all groups show a sharp reduction in the rate of calcium accumulation after the first two minutes (Figure 3.3), when calcium accumulation plateaus for all groups studied. Generally, under these conditions the calcium ATPase activity continues despite the decreased calcium uptake, indicating no impairment of the calcium pump, and therefore calcium influx (Feher and Lipford, 1985). The most probable cause for the plateau effect is an increased calcium efflux to match calcium influx (Kirchberger and Wong, 1978). The exact mechanism for this increased calcium efflux is unknown, although it is not due to calcium release through the ryanodine sensitive channel, as addition of this drug will increase the amount of SR calcium accumulated, but it will not eliminate the plateau effect. Feher and Lipford (1985) believe the plateauing of the calcium uptake is due to distortion of the SR membrane by calcium oxalate or calcium phosphate crystals, causing calcium to be lost from the lumen, and uncoupling calcium uptake and calcium ATPase. They note that due to this physical limitation of the SR membrane, the level of calcium which can be accumulated within the SR vesicles is generally related to the volume of the SR vesicles. For the data presented here, the increased level of calcium accumulation in the hibernating state (Figures 3.3 - 3.6) indicates an increase in SR volume is occurring in this state. As calcium accumulation by SR is limited by the back inhibition of calcium on the calcium ATPase pump protein (de Meis, 1981), and calcium concentration is a function of volume, an increase in SR volume will lead to a greater level of calcium storage in

vivo. The level of calcium accumulated here, in the absence of ryanodine, represents oxalate supported calcium storage into nonjunctional SR.

The addition of 0.5 mM ryanodine to the calcium accumulation medium increased the total amount of calcium accumulated in all groups (Figure 3.8). Ryanodine is thought to achieve this effect by blocking calcium efflux from a subpopulation of SR vesicles, namely junctional SR (Besch, 1985). Recently, tritiated ryanodine has been found to bind with "Feet Structures", a 340,000 Da protein in both skeletal and cardiac muscle (Imagawara et al., 1987; Inui et al., 1987; Lai, 1987). This protein is associated with the terminal cisterna-T tubule junction, and is found in preparations of "heavy" SR which are used in calcium release studies (Meissner, 1986; Meissner et al., 1986; Meissner and Henderson, 1987). The ability of ryanodine to increase calcium accumulation in all of our preparations indicates we have a heterogeneous SR preparation containing both nonjunctional and junctional SR, and suggests the presence of the feet structure protein (ryanodine receptor) on junctional SR in ground squirrels. The similar extent to which ryanodine stimulated calcium uptake by junctional SR in all groups (Fig. 3.8) indicates that the volume of this type of SR was similar for all groups (hibernating, spring, summer and fall). This is in contrast to the volume of nonjunctional SR, as measured by calcium accumulation in the absence of ryanodine, where the hibernating group had the greatest volume of nonjunctional SR. These results indicate that the nonjunctional SR is affected most by hibernation. The similar volumes of junctional SR also indicate that the SR isolation procedure

was not biased for any single group, and that bias could not account for the increased activity, both calcium ATPase and calcium uptake, in the hibernating group.

Our observations agree with those of Kondo and Shibata (1984) in that the addition of ryanodine to the reaction medium inhibited calcium efflux from junctional SR by increasing, in our study, the amount of calcium which the SR preparations can accumulate. Saxon (1986) reports that the addition of ryanodine to arctic ground squirrel papillary muscle preparations increased calcium efflux from SR. This discrepancy in the effect ryanodine has on papillary muscles seen between Kondo and Shibata (1984) and Saxon (1986) can be explained by the concentration of this drug used in each of the studies. Saxon uses a concentration of 3 X 10^{-8} M, while Kondo uses a concentration of 2 X 10^{-6} M. The low concentration of ryanodine used by Saxon increases calcium efflux from junctional SR (Hunter et al., 1983; Saxon and Kobrinski, 1988), while the higher concentrations used by Kondo and Shibata, and in this study, can block calcium release from SR (Sutko and Willerson, 1980; Alderson and Feher, 1987).

The high calcium accumulation by the nonjunctional SR in the hibernating state results in a lower percentage increase in calcium accumulation when ryanodine is added to the reaction medium (Fig 3.7). As the nonjunctional SR functions in calcium uptake (Chu et al., 1986), and the junctional SR functions in calcium release (Jones et al., 1986), these results suggest that an imbalance in the ratio of calcium accumulation to calcium release occurs during hibernation. This implies that increased calcium accumulation is required during hibernation,

while the release component is adequate for the increased levels of calcium the SR is accumulating and releasing as proposed by Kondo and Shibata (1984).

Although the hibernating group had the highest rate of calcium uptake in the calcium electrode method, the fall group gave results that ranged between the hibernating and winter/spring groups. In the $^{45}Ca^{2+}$ method, the fall group had a level of calcium accumulation greater than summer and spring animals at higher temperatures 37 and 28°C, and greater than summer animals at 19 and 10°C. These results suggest that, in the fall animals, modification of the SR begins before the animals disappear underground for hibernation in mid October. The low activity in the spring and summer groups indicates that the changes in SR activity which occur during hibernation are lost soon after emergence from the burrows in the spring and that SR activity remains low during the summer. The results are unlikely to be related to the time of year in which the animals were killed, in that winter animals, killed during the same season as the hibernators, had activities as low as the other nonhibernating groups (Figures 3.1 and 3.2).

In summery, the increased cardiac SR calcium uptake, and the increased level of calcium accumulation, seen in the hibernating group would allow the animal to recirculate more activator calcium for the increased contractility seen in the hibernating state. The ability to reaccumulate the calcium quickly is advantageous considering the harmful effects of excess calcium on cell metabolism. The volume of junctional SR was similar for all groups studied indicating that SR isolation was not biased for any one group, eliminating this hypothesis as a possible

reason for the increased activity seen in the hibernating group. Hibernation selectively increases the activity/volume of the nonjunctional portion of the SR, which functions primarily in calcium reaccumulation, emphasizing the importance of this SR function in hibernation. The increased SR activity in the fall state, over other nonhibernating groups, indicates that modifications to the SR occur before the animals disappear underground for hibernation.

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IV. SEASONAL VARIATIONS IN SR CALCIUM TRANSPORTING EFFICIENCY.

A. Introduction

Hibernation is a strategy for saving energy during winter when food supplies are scarce (Wang, 1978). During deep hibernation, at low body temperatures, a decrease in ATP concentration has been observed in heart tissue in some hibernators (Willis, 1982). If energy supplies are limited at low temperatures, then it would be advantageous for the organism to obtain the most efficient use of that energy. With sarcoplasmic reticulum it is widely believed that a stoichiometric ratio of 2 calcium transported per ATP molecule hydrolysed is the maximum efficiency obtainable from the 100,000 Da protein calcium pump (Tada et al, 1978; de Meis and Vianna, 1979; Hasselbach, 1981; Meltzer and Berman, 1984; Berman and Meltzer, 1985). This stoichiometric ratio has also been observed when the pump reversed, synthesising 1 ATP for every 2 calcium effluxed from the SR lumen though the calcium ATPase pump protein (Hasselbach, 1978). Coupling ratios of less than 2 have been obtained in SR preparations from skeletal muscle (Meltzer and Berman, 1984), and coupling ratios of 1 or less are frequently obtained in canine cardiac muscle SR preparations (Jones et al., 1979; Chamberlain et al., 1983; Chamberlain et al., 1984). In rodent species coupling ratios around 0.11 to 0.16 Ca/ATP are obtained from isolated rat cardiac SR preparations (calculated from Penpargkul et al., 1981 and Ganguly et al., 1983 respectively, and Pehowich D.J., personal communications). The reason for lower than theoretically expected coupling ratios is currently unknown. In this study coupling ratios from hibernating and nonhibernating Richardson's ground squirrels were examined to determine

if a change in the efficiency of the SR calcium accumulation occurs when the animal enters hibernation, and if this change supports the theory of energy conservation during hibernation at the molecular level.

B. Materials and Methods.

Animals and SR Preparation

The animals used in this experiment, and the method for the isolation of cardiac SR microsomal fractions were the same as those used in Chapter 2.

Combination of the Calcium Electrode and Inorganic Phosphate Methods

Calcium accumulation was followed using the calcium electrode method as described in Chapter 3, and calcium ATPase activity was monitored using the inorganic phosphate method as described in Chapter 2. Oxalate was used as the SR calcium trapping anion in this study. The reaction mixture for this experiment was of the same composition as that used in the calcium electrode method. The calcium electrode was calibrated using two 100 nmol additions of $CaCl_2$. Temperature regulation was achieved by employing a circulating water bath and jacketed glass vessel. The reaction was started by the addition of 100 ug of SR protein to a reaction mixture volume of 5 ml. Immediately after the addition of the SR protein to the reaction mixture 1 ml was withdrawn and added to a test tube containing 1 ml of cold R-III and placed on ice. The amount of inorganic phosphate contained in this sample was the time = 0 minutes sample, and was subtracted from all subsequent samples withdrawn from the reaction mixture. When a 1 ml

sample was removed for inorganic phosphate analysis the time was noted on the chart recorder for determination of the amount of calcium accumulated by the SR. The reaction was repeated with calcium being replaced by 1 mM EGTA to determine the basal ATPase activity. The amount of calcium accumulated by the SR was divided by the amount of inorganic phosphate produced to determine the coupling efficiency of the SR.

Combination of the $^{45}Ca^{2+}$ and NADH Linked Enzyme Methods.

Calcium accumulation was monitored using the $^{45}Ca^{2+}$ method as described in Chapter 3, and calcium ATPase activity was monitored using the NADH linked enzyme assay as described in Chapter 2. Inorganic phosphate is used as the calcium trapping anion in this assay as oxalate is inhibitory to the lactic dehydrogenase enzyme. Because the calcium uptake and calcium ATPase assays could not be run simultaneously, the cardiac SR used for these assays was divided into aliquots after isolation and frozen so that the experiments could be run in series. The NADH enzyme linked assay was conducted first to determine a time course for the calcium ATPase activity for each preparation. $^{45}Ca^{2+}$ was studied using the same reaction mixture including NADH and LDH enzyme (although these enzymes were not critical for ATP regeneration). The time course of the SR $^{45}Ca^{2+}$ accumulation was tailored to the time course obtained from the calcium ATPase assay. The first three time points were held constant for all groups (30 seconds, 1 minute and 2 minutes at 37°C, and 1,2 and 5 minutes at 19°C), and an aliquot was filtered at the mid point of the run, and a final aliquot was taken near the end of the

run. Accumulation of $^{45}Ca^{2+}$ at each time point was determined by filtering two separate samples. Temperature was controlled in the NADH linked enzyme assay by using a circulating water bath to pump water through the cuvette holder, and in the $^{45}Ca^{2+}$ method by means of a circulating water bath and a jacketed vessel. The coupling ratio was determined by performing a least squares linear regression on the linear portions of the calcium ATPase activities and calcium accumulation data and dividing the slope of the calcium accumulation by the slope of the calcium ATPase for a specific sample of SR. Each linear regression was performed on a minimum of 4 time points.

A t-test was conducted to determine if the coupling ratios calculated for the different groups, for each of the methods, were significantly different from each other ($p < 0.05$).

C. Results

The results of the coupling ratio experiment using the calcium electrode method to measure calcium uptake and the inorganic phosphate method to measure calcium ATPase activity are shown in Table 4.1. Of the four groups studied: hibernating, spring, fall, and winter, the hibernating group had a significantly higher Ca/ATP ratio than the other three groups at both 37 and 19°C. There was no difference between the spring and fall groups at either temperature. The Ca/ATP ratio of the winter group was significantly greater than that of the spring or fall group at 37°C, but only greater than the spring group at 19°C. Each group showed an increase in coupling ratio when temperature was

Table 4.1 The coupling ratios determined by using the calcium electrode and the inorganic phosphate methods to measure calcium uptake and calcium ATPase activity, respectively. The hibernating group had the highest coupling ratio at both 37 and 19°C (double star both temperatures, $p < 0.05$). The winter group had a higher coupling ratio than the fall and spring groups at 37°C (star, $p < 0.05$), but was only greater than the spring group at 19°C (star, $p < 0.05$). Shown is mean $\pm$ standard error.

COUPLING RATIO

	n	37°C 4 MINUTES	19°C 10 MINUTES
HIBERNATING	6	0.220 ± 0.015**	0.329 ± 0.020**
SPRING	5	0.108 ± 0.013	0.123 ± 0.018
FALL	6	0.109 ± 0.011	0.165 ± 0.018
WINTER	6	0.152 ± 0.010*	0.210 ± 0.022*

decreased.

The results of the coupling ratio experiment using the $^{45}Ca^{2+}$ method to measure calcium accumulation and NADH linked enzyme method to measure calcium ATPase are shown in Table 4.2. In this experiment the winter group was omitted and a summer group was added. Although the hibernating group had the highest Ca/ATP ratio of all groups studied, it was only significantly greater than the summer group. The coupling ratios measured in this study were higher than in the previous study. Unlike the previous study however, they showed a decrease rather than an increase in coupling ratio when temperature was reduced. The reason for this is unknown, although it may be associated with the use of different calcium precipitating anions, namely oxalate which is used in the first study, and phosphate which is used in the later study.

D. Discussion

The Ca/ATP coupling ratio of cardiac SR activity is the most difficult parameter of SR activity to study as variations in calcium accumulation, in conjunction with variations in calcium ATPase activity, can produce a large change in the Ca/ATP coupling ratio. From the two studies conducted into the coupling efficiency of the SR it can be concluded that the hibernating group has a more efficient SR (first study using oxalate as the calcium trapping agent), or is at least as efficient as some of the nonhibernating groups (fall and spring, second study using inorganic phosphate as the trapping agent), and more efficient than others (summer group). The coupling ratios presented for

Table 4.2 The coupling ratio calculated using the $^{45}Ca^{2+}$ and the NADH linked enzyme method to measure calcium uptake and calcium ATPase activity, respectively. Although the hibernating group had the highest coupling ratio, it was only statistically greater than the summer group at both temperatures ($p < 0.05$, as represented by the star). The spring and fall groups were not significantly greater than the summer group. Shown is mean $\pm$ standard error.

COUPLING RATIO

	n	37 °C Ca/ATP	19 °C Ca/ATP
HIBERNATING	4	0.337 ± 0.031	0.273 ± 0.015
SPRING	4	0.256 ± 0.034	0.253 ± 0.036
SUMMER	7	0.206 ± 0.027*	0.167 ± 0.019*
FALL	6	0.287 ± 0.031	0.226 ± 0.024

both methods, while being much lower than the ideal ratio of 2 Ca/ATP, are close to values found within other rodent species (values of 0.11 and 0.16 Ca/ATP as calculated from Penpargkul et al., 1981 and Ganguly et al., 1983 respectively). The increased coupling ratio observed at 19°C in the first method (Table 4.1), using oxalate as the calcium trapping anion, agrees with the results of Meltzer and Berman (1984) who also used oxalate, however the decrease in coupling ratio from 37 to 19°C seen in all groups when inorganic phosphate was used as the calcium trapping anion (second method, Table 4.2) is contradictory to the results of Meltzer and Berman. The reason for the decrease in coupling ratio as temperature is decreased in the second assay is unknown, although we note that no other study on the effects of temperature on coupling ratio has ever been performed using inorganic phosphate as a calcium trapping anion. Despite the decrease in coupling ratio in all groups in the second method, the hibernating group has the highest coupling ratio at each temperature (although not significantly higher than the spring and fall groups).

The relatively high coupling ratio seen at 37°C in the hibernating group is expected since the high rate of calcium uptake observed in this group (Chapter 3) is not matched by an equally high calcium ATPase (Chapter 2, Figure 2.4). Although at 19°C, the calcium ATPase activity for the hibernating group is increased in relation to the nonhibernating groups (Chapter 2, Figure 2.6), the hibernating group still has the highest coupling ratio at this temperature, whether significantly higher than the other groups or not. This result indicates that the rate of calcium uptake in the hibernating group, when compared to the

nonhibernating groups, at 19°C is even greater than would be seen at 37°C. If the ratios of the amount of calcium accumulated in the presence of oxalate for the hibernators in relation to the nonhibernator groups at 19°C for the 10 min mark (see Figure 3.5 of Chapter 3: 2.92 fold over spring; 4.28 fold over summer; 3.51 fold over fall) are compare that with the ratio at 37°C for the 5 min time point (see Figure 3.3 of Chapter 3: 2.72 fold over spring; 2.80 fold over summer; 2.20 fold over fall) it is obvious that even though the calcium ATPase activity is increasing at 19°C the amount of calcium being accumulated is also increasing in proportion to nonhibernators at these time points (time points which roughly correspond to the times at which coupling ratios were determined in the first method using oxalate as a calcium trapping anion). This demonstrates that while an increase in calcium ATPase activity occurs in hibernating animals relative to nonhibernating animals at 19°C, the rate of calcium uptake is also increased in hibernating animals to maintain the high coupling ratio seen at this temperature relative to the nonhibernating groups.

The higher coupling ratio seen in the hibernating group indicates a more efficient SR. This would be advantageous at lower temperatures as levels of ATP have been reported to decrease in some hibernating species (Willis, 1982). This implication should however, be viewed with caution as the reason for the low coupling ratios in rodents is currently unknown. Additionally, while a slight increase in coupling ratio could result in some energy savings during hibernation, the total amount of energy saved can only be determined by studying the SR Ca/ATP ratio under the *in vivo* conditions over which the SR must function

during hibernation (ie. we do not know how the coupling ratio is maintained over a calcium concentration range, or possible changes in intracellular pH; all of which have been known to influence the coupling ratio (Meltzer and Berman, 1984)).

The reason for a low or variable coupling ratio is unknown. Hasselbach (1981) suggests that the reason for a coupling ratio of less than 2 is due to leaky SR vesicles, although Meltzer and Berman (1984) found no evidence of this when they titrated a population of skeletal muscle SR vesicles with calcium and limited amounts of ATP. When the ATP had been exhausted the amount of calcium effluxing from the SR remained low despite the concentration gradient between the SR and the external medium which had built up through the active accumulation of calcium. These authors concluded that coupling ratios lower than the ideal state (ie. Ca/ATP = 2) are most likely due to slippage of the calcium pump as the calcium ATPase passes through its enzyme cycle, indicating an efflux or loss of calcium from the lumen of the SR is via the calcium pump. Thus, the lower coupling ratios they obtained cannot be explained by a simple leakage of calcium from the SR vesicles as proposed by Hasselbach, but rather involves a loss of calcium through a complex reaction cycle of the calcium ATPase. Berman and Meltzer (1985) suggest that the coupling of SR calcium uptake to calcium ATPase is closely related to phosphorylation of phospholamban. This idea follows the suggestion of Shamoo and Ambudkar (1984) who propose that both the calmodulin and cAMP dependent phosphorylation sites on phospholamban in cardiac SR need be phosphorylated for SR to accumulate calcium. Thus the higher coupling ratio seen in the hibernating group

could reflect a greater modification of the SR through increased phosphorylation of phospholamban in this group.

Another explanation for the high coupling ratio seen in hibernating animals, and not exclusive of the hypothesis put forward by Berman and Meltzer (1985), is the variable activity of the nonjunctional SR in relation to the junctional SR in hibernating animals seen in Chapter 3. The coupling ratio can be improved by the addition of SR calcium release inhibitors such as ryanodine to the calcium uptake solution (Chamberlain et al., 1984), indicating that calcium efflux from junctional SR release channels leads to a lower observed coupling ratio (it should be noted however, that Ryanodine treatment alone will not increase the coupling ratio to ideal values). It can therefore be stated that the coupling ratio is independent of the amount of SR present in a preparation, but not independent of the type of SR in the preparation. An increase in longitudinal SR, either in amount or activity (ie. for calcium uptake), without a corresponding increase in junctional SR (which has calcium efflux through release channels) would result in a higher coupling ratio. In the hibernating preparation the observed coupling ratio in each experiment was highest (Tables 4.1 and 4.2). An increase in calcium accumulation by nonjunctional SR was observed in the hibernating group without a corresponding increase in junctional SR (as expressed by the amount of calcium accumulated in the presence of ryanodine, see Chapter 3). This differential variation in SR activity between the junctional and nonjunctional SR could be responsible for the increased coupling ratios observed in the hibernating state.

In summary, although the coupling ratios were lower than the ideal state of 2 Ca/ATP, the hibernating group had the highest coupling ratio, for each of the two studies conducted, at both 37 and 19°C. The reason for the increased coupling ratio observed in the hibernating group (especially in the presence of oxalate) in comparison to the nonhibernating groups is presently unknown. According to a theory put forward by Meltzer and Berman (1985), it could be due to an increase in the number of phosphorylated regulatory proteins in the hibernating preparation. A change in the ratio of the activity of longitudinal vs. junctional SR could also be responsible for this increase. Further work in measuring the activity of separated nonjunctional and junction SR will provide information on how these different types of SR affect the overall coupling ratio in a combined SR fraction.

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V. GENERAL DISCUSSION

Mammalian hibernation represents a unique physiological condition in which the animal must survive low body temperatures for a prolonged period of time, and retain the ability to rewarm to 37°C without the aid of endogenous heat. It is therefore not surprising that a superior cold tolerance of many physiological processes have been observed in hibernators when compared to nonhibernators (Lyman and Blinks, 1959; Marshal and Willis, 1962). Electrical stimulation of papillary muscle preparations from hibernating hamsters and Richardson's ground squirrels resulted in increased tension generation in comparison to animals in the nonhibernating phase (South and Jacobs, 1973; Zhou et al. unpublished observations). In the hibernating Richardson's ground squirrel papillary muscle preparations, the increased tension development is accompanied by a decrease in the time taken to reach peak tension, indicating that the rate of tension rise is greater in these animals. Analysis of the data of Zhou et al., with respect to the rate of papillary muscle relaxation, indicates a greater rate of relaxation in the hibernating phase at lower temperatures (see Appendix A). The results suggest a greater amount of activator calcium is present in the hibernating cardiac myocyte during contraction, and that this calcium is removed from the sarcoplasm at a greater rate than in the nonhibernating state. As the mechanical activity of a muscle is related to changing concentrations of activator calcium during contraction and relaxation, these results suggest that changes in calcium metabolism are occurring in hibernation. Since the sarcoplasmic reticulum is the most important organelle involved in striated muscle calcium metabolism (Carafoli,

1985), and SR activity correlates positively with force generation in muscles (Ganguly et al., 1983; Tate, 1986; Van Winkle, 1986), the results presented above suggest that an increase in SR activity is occurring during hibernation.

Cardiac sarcoplasmic reticulum was isolated from myocardial tissue obtained from animals captured at different times of the year (spring, summer and fall), and during the hibernation season (Oct. - March) from animals kept in captivity which were induced to hibernate (hibernating group), or which were physiologically unprepared to hibernate (winter group). The hibernating group had the highest intrinsic calcium ATPase activity as evidenced when calcium accumulation and calcium ATPase were uncoupled by the addition of A23187 to the reaction medium (Chapter 2). While the calcium ATPase activity of the hibernating group, in the absence of A23187, was not significantly greater than that on the nonhibernating groups at 37°C it was significantly greater at 19°C (Figs. 2.4 and 2.6), suggesting a lower thermal sensitivity and emphasising the functional importance of this enzyme at lower temperatures in hibernators. The rate of calcium uptake is also highest in the hibernating animals over the physiological temperature range that they must survive during hibernation (Chapter 3). Oxalate-supported calcium accumulation is also greatest in the nonjunctional SR of the hibernating animals suggesting an increased SR volume for calcium storage (Figs. 3.3-3.6). Finally, the hibernating group showed somewhat higher Ca/ATP coupling ratio at 37 and 19°C, suggesting a more efficient use of energy for Ca^{2+} transport (Chapter 4, Tables 4.1 and 4.2). This may be important if energy stores become limited during hibernation.

Taken together these observations are consistent with the seasonal variations seen in the mechanical activity of the papillary muscles suggesting that changes in SR activity were occurring during hibernation. The SR activities documented in the present study provide the cellular mechanisms for the observed increased rate of relaxation seen in the hibernating state (Appendix A).

The increased SR calcium accumulating activity in the hibernating state suggests, as does the increased tension development, that cardiac myocytes in this state could be experiencing a higher level of intracellular calcium during excitation. An increased SR activity would be expected as Kondo and Shibata (1984) suggest that during hibernation cardiac myocytes rely more upon the internal recycling of calcium through the SR and less upon extracellularly derived calcium for contraction. The increased reliance upon SR as a source of calcium for excitation-contraction coupling, and the increased mechanical activity, suggest a greater calcium release from SR during hibernation. A greater release of calcium from SR would reduce the need for extracellular calcium for contraction. Internal cycling of large amounts of calcium in cardiac myocytes occurs in hibernating species such as the european hedgehog (Duker et al., 1986). The authors suggest that the shortened plateau phase (ie. slow calcium current) of the cardiac action potential, seen in the hedgehog, is due to an increased level of intracellular calcium which is known to shorten the duration of the action potential (Isenberg, 1975). This finding is similar to that observed by Kondo (1987) in the hibernating chipmunk, in which he proposes that the shortening of the action potential in this species is

the result of an activation of the Na-Ca exchanger in the sarcolemma due to a greater level of intracellular calcium released from internal stores. The shortening of the action potential would result in less extracellular calcium entering the cell, causing a greater emphasis to be placed on the SR as a source of calcium for excitation-contraction coupling. Our results would suggest that this increased level of intracellular calcium, which occurs during excitation-contraction coupling in the hibernating myocardium, could be accommodated by an increased rate of SR calcium uptake and calcium storage, both of which would be required for a greater release of calcium from SR. Indeed, as the amount of calcium available within the SR for release is related to the amount which the SR can accumulate (Blinks, 1986), the increased calcium uptake by SR would make more calcium available for release.

In relation to the possible increase in the level of intracellular calcium in hibernating cardiac myocytes during excitation-contraction coupling, Kondo (1986) suggests that the hibernating myocardium functions as though it is in a calcium overloaded state. This is based upon his observation that the transient increase in contractility caused by the addition of ryanodine to the papillary muscle is similar to that seen in calcium-overloaded purkinje fibers (Valdeolmillos and Eisner, 1985). In the case of the hibernators the term "overloaded" refers only to the ability of the SR to release calcium, not to a cardiac myocyte which has a pathologically high level of intracellular calcium which is causing a deleterious effect on myocardial function (Dhalla, 1984). A similar suggestion was also made by Saxon (1986) who noted that hibernating papillary muscle preparations would not respond to positive

inotropic stimuli by increasing force production, while preparations from nonhibernating animals would. It would appear from these observations that, in the hibernating state, the SR is not only storing and releasing more calcium than in the nonhibernating state, but that the capacity of the SR to release calcium for contraction is functioning at a maximum level.

To explain the variations in the behaviour of papillary muscles from nonhibernating and hibernating chipmunks, Kondo (1986) utilizes a two compartment theory for the cycling of intracellular calcium (Kaufmann et al., 1974; Allen et al., 1976; Edman and Johannsson, 1976). In this theory two intracellular compartments are responsible for calcium accumulation and calcium release, respectively. It is believed that compartment I represents the nonjunctional SR, and that compartment II represents the junctional SR (Edman and Johannsson, 1976; Schouten et al., 1987). Calcium is released from compartment II during muscle excitation to bind with troponin C leading to a muscle contraction. Most extracellular calcium entering the muscle cell during the action potential, as well as calcium released from compartment II is accumulated by compartment I leading to muscle relaxation. During rest, calcium is transported from compartment I to compartment II for release during subsequent depolarizations. To balance the system, and to prevent an excess of calcium accumulation within the cell, calcium can be removed from compartment II during rest and transported from the cell at the SL membrane through a Na^{+}-Ca^{2+} exchanger and SL calcium ATPase. Kondo (1986) suggests that the transport of calcium from compartment I

to compartment II is saturated in hibernation, giving the cardiac myocytes the appearance of being calcium overloaded.

Our results on oxalate-supported calcium accumulation in nonjunctional and junctional SR suggest that nonjunctional SR activity is being selectively increased in the hibernating state, creating a change in the proportion of calcium accumulating to calcium releasing SR (Chapter 3). In could be hypothesised that an increase in the calcium uptake (nonjunctional SR) component of the SR, without a subsequent increase in the release component (junctional SR), could lead to an overload in the junctional SR as more calcium from the nonjunctional SR is available to be moved into the junctional SR during muscle relaxation. This would have the effect of increasing the amount of calcium available in SR for release, and at the same time overloading the release compartment as it does not increase proportionally in volume with the uptake compartment.

This hypothesis would also provide the basis for explaining the decreased response of hibernating papillary muscles to positive inotropic stimuli such as noradrenaline and isoproterenol (Charnock et al., 1980; Kondo, 1986; Saxon, 1986; Senturia et al., 1986). Positive inotropic compounds, such as isoprenaline and noradrenaline, are generally thought to increase contractility in muscle cells by increasing the amount of activator calcium within the SR available for release (Blinks, 1986). These compounds increase trans-sarcolemmal calcium influx, and increase calcium accumulation by SR, by phosphorylating phospholamban through increased levels of cAMP, which increases the rate at which the calcium ATPase passes through its enzyme

cycle. These changes lead to increased calcium loading of the SR, through increased calcium uptake, making more calcium available for release from SR, thereby increasing force generation within the muscle. The decreased response to positive inotropic stimuli seen in the hibernating state is not due to a decreased trans-sarcolemmal calcium influx as Kondo (1986) discovered that the addition of isoprenaline to hibernating papillary muscle preparations increased the slow inward calcium current, thereby increasing the amount of calcium entering the myocyte. While the additional influx of calcium potentiated contractility in the nonhibernating state, it failed to do so in the hibernating state. Kondo suggests that in the hibernating state, compartment I (nonjunctional SR) has a sufficient capacity to accumulate the excess calcium entering the cardiac myocyte as a result of the isoprenaline, because treatment of papillary muscles with this compound did not result in increased resting levels of cell calcium, which would have caused an increase in the resting tension of the papillary muscles. Our results agree with this hypothesis in that both the capacity and rate of calcium accumulation by nonjunctional SR is greater in preparations from hibernating animals in comparison to nonhibernating animals. Thus the SR in the hibernating myocytes appear to have the capacity to accumulate the extra calcium load placed on the SR through the actions of isoprenaline, even though the SR is already sequestering an increased level of calcium in the hibernating state. If the increased calcium influx caused by isoprenaline is absorbed by compartment I (nonjunctional SR), but not translated into a positive inotropic effect, then the factor which eliminates the isoprenaline

effect occurs after this event. Kondo (1986) suggests that the limiting step is the rate at which calcium is transported between compartments I and II. Saxon (1986) arrived at a similar conclusion when he observed in arctic ground squirrels (Citellus parryi) that papillary muscle preparations from nonhibernating animals responded well to positive inotropic compounds such as adrenaline and ouabain, but that hibernating squirrels respond to these agents only after treatment with low doses of ryanodine (3×10^{-8} M). The effect of the low dose of ryanodine was to deplete the SR of calcium before stimulating the muscle to contract (Hunter et al., 1983; Saxon and Kobrinski, 1988). Saxon suggests that in the hibernating state the SR responds as though it is overloaded with calcium, and that by depleting it with ryanodine the inotropic agents can increase contractility by increasing the relative amount of calcium in the SR available for release. Thus, it can be hypothesised that hibernating myocardium fails to respond, or shows a diminished response in comparison to nonhibernating myocardium, when challenged with positive inotropic stimuli because of an already calcium overloaded junctional SR (calcium releasing compartment).

The increased ability of the SR to accumulate calcium, while intrinsically important to relaxation in striated muscle, also provides a major role in maintaining intracellular calcium concentrations around 10^{-7} M at rest. The ability to actively regulate intracellular calcium concentrations is important for cell survival. For example, red blood cells from guinea pigs show a loss of potassium at low temperatures as a result of their inability to actively pump out calcium leaking into the cells (see Wang and Wolowyk, 1988 for review). The rise in

intracellular calcium triggers a loss of potassium from the cell through the Gardos channel. In RBCs from hibernating Richardson's ground squirrels this potassium loss does not occur due to the ability of the cell to remove passively influxing calcium by actively pumping it out. Thus, our observed increase in the ability of hibernating SR to accumulate calcium is direct evidence that cardiac myocyte survival at low temperatures can be aided by competent intracellular Ca^{2+} regulation.

Seasonal studies on cardiac SR isolated from animals at different times of the year indicate that the increased rate and capacity of calcium uptake seen during hibernation are absent in spring animals after emergence from hibernation, remain low in summer animals, and show some signs of increasing in fall animals. The calcium electrode experiment, used to determine the initial rate of calcium uptake (Chapter 3), gave an initial rate for the fall animals higher than the winter and spring animals, but lower than the hibernating animals (Fig. 3.1). As temperature was decreased in that assay the fall group demonstrated activities which were closer to the winter and spring groups. Although the $^{45}Ca^{2+}$ assay did not show a greater initial rate of calcium uptake in the fall group over the other nonhibernating groups at 37°C, the group did have a greater level of calcium accumulation after 20 minutes of incubation (Fig. 3.2). It is unknown what triggers the changes to occur in myocardial SR activity, although it is reasonable to assume, based on the increased SR activity seen in fall animals, that changes have already begun to occur before the animals disappear underground for the hibernation season.

The biochemical events responsible for the increased cardiac SR activity observed in the hibernating ground squirrels are not readily apparent. Variations in contaminating nonSR proteins in the microsomal are unlikely to have resulted in the increased SR activity in the hibernating state. Likewise, due to the similar levels of calcium accumulation by junctional SR in hibernating, spring, summer and fall animals, it appears unlikely that isolation procedures favored SR isolation in any one particular group, indicating that the measured activity of the nonjunctional SR was representative of its activity in the myocardial tissue it was obtained from. Even though the SR calcium uptake and accumulating activity of the hibernating group, and to a lesser extent the fall group, was increased in relation to the other nonhibernating groups, these results could be due to either increased de novo biosynthesis of SR, or enhancement by increased phosphorylation of phospholamban; both of which would cause increased calcium ATPase activity, and increased calcium uptake, and increased levels of accumulation (Entman et al., 1969; Kim et al., 1987).

Many physiological states affect SR calcium ATPase activity and calcium accumulation. One or more of these physiological changes could be responsible for the observed changes in SR activity during hibernation. Hyperthyroidism increases calcium uptake and calcium accumulation (oxalate supported) in cardiac and skeletal muscle (Limas, 1978; Simonides and van Herdeveld, 1986). This increased calcium uptake is also reflected in an increase in calcium ATPase activity, indicating that thyroxine proliferates the SR and/or increases the density of the 100,000 Da protein calcium pump per unit membrane. Although this

hormone appears to be a good candidate for inducing the changes in SR activity seen in the hibernating state, its seasonal activity does not match the results obtained here, and its variability among the different hibernating species make it unlikely as a common mechanism for SR changes within different hibernators. In the Richardson's ground in particular, squirrel plasma levels of thyroxine and triiodothyronine are high during hibernation and spring, but low during the summer and fall (see Wang, 1982); although during deep hibernation at low body temperatures the free level of these hormones are low. While it is possible that these hormones could be responsible for the results observed in the hibernating state, the hormone levels are increased in spring, and low in the fall, opposite to the results seen here, making these hormones an unlikely candidate for the changes in the SR observed here.

High levels of catecholamines can also produce the results seen in the hibernating group. Increased calcium uptake by SR has been observed when catecholamines were injected in vivo before isolation of cardiac SR (Panagia, et al., 1985; Kranias et al., 1985). The catecholamines are thought to act on the SR via increased intracellular levels of cAMP, leading to the phosphorylation of phospholamban (Kirchberger and Tada, 1976; Tada et al., 1979; Tada et al., 1980; Tada et al., 1983; Kranias, 1985). Phosphorylation of phospholamban increases calcium transport and calcium ATPase activity (Tada et al., 1982). Incubation of SR vesicles with cAMP increased the amount of calcium the vesicles could store as well as increasing rate of calcium uptake (Entman et al., 1969; Kim et al., 1987). This observation indicates that increased calcium

accumulation seen in the hibernating state is not necessarily due to a biosynthesis of more SR, but could be due to a modification of SR activity through phosphorylation of phospholamban. Several investigators have noted that the addition of cAMP causes a 2-3 fold increase in the rate of calcium uptake by cardiac SR (see Lindemann et al., 1986 for review), similar to the observations seen between the hibernating and nonhibernating groups (Chapter 3). It is unlikely however, that circulating catecholamines would cause this effect during hibernation, as noradrenaline and adrenaline levels are low during deep hibernation, and only increase after the animal has started to arouse from hibernation (Florant et al., 1982). It is still possible however, that phosphorylation of phospholamban, through some as yet unknown stimulus, could account for the results seen here.

In summary, we have shown that cardiac SR calcium ATPase, calcium uptake and calcium accumulation, and to some extent the Ca/ATP coupling ratio, increases in activity during hibernation. These results correlate with the increased myocardial relaxation seen in hibernating animals (Appendix A), and imply that the SR can accommodate the increased levels of intracellular calcium suggested by the increased rate and extent of tension development in papillary muscles in Richardson's ground squirrels (Zhou et al. unpublished data). These results support the hypothesis of Kondo and Shibata (1984), in which they propose an increased reliance upon the SR as a calcium source for excitation-contraction coupling occurs in the hibernating state. Certainly, the increased SR activity reported here suggests that this organelle plays an important role in myocyte calcium metabolism within

hibernating ground squirrels. The observation that an increase in the activity/volume of nonjunctional SR is not matched, in the hibernating state, by an increase in junctional SR emphasizes the importance of calcium accumulation in hibernators. This disproportionate increase in nonjunctional SR in relation to junctional SR forms the basis for a hypothesis explaining why the hibernating myocardium responds as though it were overloaded with calcium, and why this tissue shows a decreased positive inotropic response to catecholamines.

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APPENDIX A

Figure A.1 Relative decrease in the rate of relaxation of papillary muscles from hibernating and winter nonhibernating Richardson's ground squirrels as affected by temperature. Data was calculated from the unpublished work of Zhou et al. The hibernating group shows an increase in the rate of relaxation initially as temperature is decreased, and remains greater than the winter nonhibernating group at lower temperatures. Symbols represent mean values, while bars represent +1 and -1 standard error for the hibernating and winter groups, respectively. Stars represent a statistically significant difference based on an unpaired t-test ($p < 0.05$) between the two groups at a given temperature. Temperature is shown on an absolute scale (Kelvin).

RELATIVE -dF/dT AS EFFECTED BY TEMPERATURE

(FROM 1985 DATA of Zhou et al.)